

# VETERINARY TOXICOLOGY

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Second Edition

# VETERINARY TOXICOLOGY

## Basic and Clinical Principles

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Second Edition

Edited by

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*This book is dedicated to my daughter Rekha, wife Denise, and parents the late Chandra and Triveni Gupta*

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# Preface

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Veterinary toxicology is a very complex, yet fascinating, subject as it deals with a wide variety of poisons of plant, chemical and animal origins. Presently, synthetic compounds constitute the largest number of chemicals that are frequently encountered in animal poisonings. The subject of veterinary toxicology is complicated greatly by the wide variations in responses of domestic, aquatic, wild and zoo species to toxicants. Of course, there are many other factors that can be involved in the overall toxicity of a chemical. In the last few decades, veterinary toxicologists have faced the enormous task of dealing with a flood of new farm chemicals and household products. Understanding the complete profile (especially mechanisms of toxicity) of each toxicant is the biggest challenge for today's veterinary toxicologists.

By the turn of the 21st century, we began to face new problems. For example, during the incident of September 11, 2001, a large number of pets died from the collapse of the World Trade Center in New York City, while the surviving ones still suffer from a variety of illnesses (Ground Zero Illnesses) caused by the dust, debris and toxic chemicals. In 2005, two major hurricanes (Katrina and Rita) devastated the lives of so many animals in the Gulf Coast states of Louisiana and Mississippi. Thousands of animals died, while a large number of others suffered from intoxication with high levels of metals, pesticides, algal toxins and many other unidentified toxic substances.

Since the publication of the first edition of *Veterinary Toxicology: Basic and Clinical Principles* in March 2007, several man-made and natural disasters have occurred, which have devastated the lives of humans and animals alike in many parts of the world. For the interest of readers, a few such incidents are mentioned here in brief. In early 2007, melamine became a household name for pet owners in the U.S. because many dogs and cats died

from renal failure, while hundreds were sickened due to the consumption of food tainted with melamine and other co-contaminants (cyanuric acid, ammeline and ammeline) imported from China. Regulatory action of the U.S. Food and Drug Administration led to the recall of more than 150 brands of pet food. Similar recalls occurred in Asia and South Africa. One year later, more than 300,000 children were found to be affected in China by melamine-adulterated infant formula. Over 50,000 children were hospitalized and at least a dozen of them died. Children were also reported to be sick from melamine in other parts of Asia, including Singapore, Taiwan and Vietnam. In 2008, just prior to an international polo event in Florida, 21 horses from a Brazilian team died acutely due to an overdose of selenium. In 2010, the Deepwater Horizon oil rig explosion killed 11 workers. The subsequent oil spill in the Gulf of Mexico caused deaths or affected the lives of millions of birds, fish and other species including dolphins, costing billions of dollars. In the recent past, massive flooding in Australia, Pakistan, the U.S., and many other parts of the world has caused havoc for animals because of drowning, snake bite and toxic debris exposure. On March 11, 2011, a severe earthquake and subsequent tsunami that struck Japan led to the deaths of thousands of humans and animals. As a result of the tsunami and aftershocks, the Fukushima Daiichi Nuclear Power Plant was severely damaged. Deaths of many companion animals occurred within a few days from excess radiation exposure. A large number of surviving abandoned livestock, horses and companion animals in the danger zone continue to suffer from cancer and other radiation-related complications. One month later, a record number of tornadoes (>365) hit the U.S. in various states within a span of less than a month. Two killer tornadoes, which hit the southern part of the U.S., one in Tuscaloosa, Alabama, and the

other in Joplin, Missouri, devastated the lives of humans and animals alike.

It appears that from time to time, unusual toxicological problems are encountered on a small or large scale, and that trend will most likely continue in the future. Around the world, animals and humans are living in a more polluted environment today than ever before. Many of the toxicological problems are global, while others are regional. Unfortunately, the availability of antidotes for common poisons is still obscure and often delayed. Thus, veterinary toxicologists have a tremendous task ahead to face the new challenges of the 21st century.

The first edition of *Veterinary Toxicology: Basic and Clinical Principles* was prepared to offer a comprehensive resource to veterinary toxicologists, students, teachers, clinicians, nutritionists, animal health scientists and environmentalists. The second edition maintains the same temper, yet is more student/teacher friendly and targeted primarily for the classroom. The existing chapters have been updated, while many new chapters (some pertinent to European problems) are added, identifying new problems. The entire volume is organized in 18 sections, with a total of 104 chapters, to offer a standalone chapter on as many topics as possible. The book is heavily focused on target organ toxicity and poisons of various types and classes. Several chapters provide the latest information on problems related to industrial, environmental, aquatic, marine and zoo toxins. This volume extensively covers chapters on poisonous plants, mycotoxins and feed and water contaminants. A significant portion of the book is devoted to diagnostic toxicology that entails sample submission, basic concepts

of analytical toxicology, toxicoproteomics and microscopic analysis of feed. Finally, the book concludes with an emphasis on prevention and therapeutic measures of common poisonings.

In the last couple of years, veterinary toxicologists from many parts of the world have expressed the need for a 2nd edition of *Veterinary Toxicology: Basic and Clinical Principles*, a standard book that can provide detailed coverage of the basic and clinical principles of veterinary toxicology. In light of these basic objectives and the current world situation, the book is revised accordingly. It addresses both global as well as regional toxicological problems, and adequately offers practical solutions. On every major topic, a standalone chapter is provided that is further enriched by major references for further reading. The chapters of this book are contributed by the most qualified and well-experienced authors who are considered authorities in their respective areas of veterinary toxicology. The overarching goals of this volume are achieved by the hard work of all authors. This book represents a collective wisdom of more than one hundred authors, and offers a unique text/reference source for those involved in veterinary medicine in general and toxicology in particular.

Finally, the editor is deeply indebted to all authors for their sincere and dedicated contributions to this book. A special thanks to Mrs. Robin B. Doss and Ms. Michelle A. Lasher for their technical assistance in the checking of references. The editor and the authors offer their sincere thanks to the publishing editors (Mary Preap and Kristine Jones) and the project manager, Julia Haynes at Academic Press/Elsevier for their vital input in the preparation of this book.

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# Veterinary toxicology: a historical perspective

Frederick W. Oehme

What's this about veterinary toxicology? Where did it come from? How did it evolve to get that way? What's its current focus and where is it going? These are all stories worth telling. Let's start at the beginning...

Animals have traditionally been "second class citizens" to humans in the hierarchy of life on this planet. They have been used as food sources, to perform labor, for sport, and in recent centuries to serve as guardians of property. Of late some species have become companions to humans and given pleasure to their owners. Their economic value increasingly caused some humans to address the health needs of animals in their care first by addressing obvious concerns, then by applying common sense and with the advent of "therapies" to use counter-measures to "heal" the stricken animal.

In the beginning folk medicine was employed to deal with sufferings produced by "spirits" or other mysterious agents. Equally vague efforts to deal with these little understood illnesses evolved into ritualistic "medicine"; the application of rituals, incantations, charms, liquids, plants and herbs. Studies of how illnesses work and categorizing them into specific etiologic agents produced medical diagnoses, and then disciplined further into pathophysiology and therapeutics for effective therapy. Individuals with the interests and experiences in medicinal chemistry observed and recorded the effects and values of such applied information.

With the use and application of natural and synthetic chemicals to reverse the ill-effects from challenged body functions, pharmacology grew into its own specialty dealing with using these same compounds for therapeutic purposes. With even more sophistication, recognition of the numerous dangerous effects from these products called for a clearer definition and understanding of what

separated a "safe" medicinal from one that caused more pain and danger than the illness being treated; i.e., the thesis of "dose is everything" was born... and with it and its confounding parameters, toxicology was born!

And what a prodigious science that birth produced! Spurred on by the industrial revolution, urbanization and "better living through chemistry," the unfortunate ill-effects of "too much" became obvious. Instances of chemical misuse, finding chemicals in unexpected locales, and the lack of the knowledge needed to deal with environmental, human and animal toxic misadventures spurred public outcries. Government actions followed, but were also led by concerned and inquiring scientists. Their recognition that there is strength and opportunities in bonding together to encourage and share the development of this necessary and energetic science flowed into the growth of international organizations fostering and sponsoring the growth of toxicology in all its important ramifications.

## THE EMERGENCE OF VETERINARY TOXICOLOGY

Increased sophistication of human pathology and forensic medicine stimulated similar developments in veterinary medicine. With the ever-increasing expansion of livestock populations on western ranges, concern developed for the death losses from consumption of poisonous plants. Poor grazing conditions and access to often unrecognized toxic range plants stimulated a wide range of investigations by federally employed as well as

private veterinarians and resulted in large numbers of publications in the 1920s and 1930s to inform livestock owners and reduce losses. With this came the need for clearly identifying what toxic components were present and how they might be effectively treated. Such studies recognized the value of the veterinarian's training and utilized his multifaceted skills in toxicologic studies for clinical evaluations. This was rapidly recognized by industrial institutions and the veterinarian's talents were sought for application to commercial drug trials in laboratory animals. These veterinary scientists wearing the hat of toxicology began expanding their responsibilities to biochemical separations, electron microscopy and studies of the cellular and molecular mechanisms of these chemical actions.

World War II and the extensive use of insecticides for pest control and speculative gas warfare resulted in veterinarians being employed by the armed forces in other experimental animal studies. After the war, these and newer chemicals were widely applied to problems in agriculture. Their use required skilled veterinary supervision and all too quickly veterinary treatment when the misuse of these potent chemicals occurred. Facilities at universities, such as Texas A & M, and at governmental research institutions, such as the Poisonous Plant Research Laboratory at Logan, Utah, focused efforts on the growing hazards and clinical problems resulting in domestic animals. The growth of the pesticide field, coupled with the intensive land use encroaching on plant and animal habitat, required increasing chemical and biological knowledge to understand and identify the disease processes involved.

Veterinary pathologists, such as C.C. Morrill, W.L. Sippel, K. McEntee and P. Olafson, became increasingly interested in the toxic problems now being seen in expanding numbers. J.L. Shupe concerned himself with detailed studies of fluorine intoxication associated with industrial pollution problems. Veterinary pharmacologists began to investigate specific toxicants and their effects on domestic animals; W.G. Huber studied toxic effects of chemotherapeutic agents and antiseptics; R.P. Link identified dicumarol as the anti-clotting factor in sweet clover poisoning and spoke out warning against insecticide poisonings; P.B. Hammond investigated heavy metal toxicities with particular interests in utilizing chelating agents to treat lead poisoning; O.H. Muth studied trace minerals and their interactions in animal intoxications; R.D. Radeleff worked extensively with insecticides and their harmful effects in domestic animals; J.S. Palmer worked closely with Radeleff performing similar investigations on herbicide and pesticide toxins; W. Binns and J.W. Dollahite studied the pathology and biochemistry of numerous poisonous plant intoxications in livestock. Information describing the specific pathology and biochemistry produced by the

increasingly recognized number of xenobiotics and naturally occurring materials were coupled with the veterinary experiences discovered in diagnosing clinical cases and effectively providing treatment.

By the mid-1950s, toxicology was a highly active area of veterinary medicine. Biochemical and molecular interactions were discussed and the tools of other disciplines were brought to focus upon the problems of domestic animal poisonings. With it, a new breed of veterinarian emerged. The developing veterinary toxicologist had to understand physiology and pathology, but equally important he had to be a chemist with wide knowledge of separative and quantitative instrumental techniques. Professional judgment of clinical episodes and a working knowledge of metabolic and excretory processes were needed. He had to become intimately familiar with pharmacology and the molecular action of a wide variety of chemicals. Understanding treatments to be administered for specific intoxications was necessary. Finally, he had to logically and scientifically put into perspective the often confusing and confounding assortment of signs, lesions and analytical results to reach rational interpretations and conclusions for the numerous problems being solved. Since increasing knowledge was being sought, this well-grounded veterinarian had also to be able to conduct significant independent research in well-equipped facilities. More and more veterinarians now were conducting toxicological research investigations as their primary mission.

## PROFESSIONAL ORGANIZATION AND ACADEMIC RECOGNITION OF VETERINARY TOXICOLOGY

In the presence of this increasing need and professional situation, a small group of veterinarians specializing in toxicology united to focus attention on the needs of veterinary toxicology and to assist the progress and growth of this discipline. The formation of the American College of Veterinary Toxicologists (ACVT) in 1958 was the beginning of formal development and recognition of veterinary toxicology. At a meeting in Salt Lake City, Utah, on January 15, 1958, the ACVT was formed by 11 veterinarians stimulated by and engaged in toxicology. The organizing committee consisted of Drs. Chapman, Christofferson, Furgeson, Harris, Hayden, Holmes, Jones, Phelps, Shupe, Spencer and Vinsel. The group's objectives were: "To further the educational and scientific progress in veterinary toxicology and to encourage education, training and research in veterinary toxicology; To establish standards of training and experience for... specialists in veterinary toxicology; To further

recognition of such qualified specialists...; To arrange meetings to promote discussion and interchange of ideas in the following fields of veterinary toxicology: teaching, research and development, diagnosis, nomenclature, public health...; To provide all possible aid and assistance to its members by the interchange of ideas and scientific information; To review manuscripts...; To review published material and keep a file on such reviews...; To accumulate and disseminate information in the field of veterinary toxicology...; To encourage adoption...of uniform clinical and laboratory reporting methods...; To suggest or direct basic research in those areas of deficient knowledge..." (Constitution, 1958).

By 1968 the ACVT grew to over 100 Fellows and Associate Fellows. It had worked efficiently and had stimulated national and international recognition of veterinary toxicology as a progressive and dynamic specialty.

This vitality was further stimulated in 1964 by the New York Academy of Sciences publishing a volume devoted to veterinary toxicology based on the proceedings of an international meeting held in New York City (Gabriel, 1964). This symposium provided basic information on the energetic activities in veterinary toxicology at that time and had the effect of stimulating further growth and multidisciplinary efforts in the field.

The increasing demand for specialized training in veterinary toxicology also encouraged academic training programs. Early efforts were established in universities at Cornell, Utah State, Iowa State, Florida, Kansas State and others. This proliferation has continued with training centers established in other universities and institutions, in veterinary diagnostic laboratories and including research training in molecular and genomic toxicology investigations throughout the United States and around the globe. These early centers and their offsprings have fostered the talents to understand and deal with numerous environmental and clinical problems in veterinary medicine.

Academic recognition of veterinary toxicology was initiated with the American Board of Veterinary Toxicology (ABVT) being formally recognized by the American Veterinary Medical Association (AVMA) in mid-1960. Largely through the efforts of R.D. Radeleff during his term as president of the ACVT, an application for approval of the specialty was accepted by the AVMA Council on Specialty Organizations. A Certifying Board of W. Binns, J.W. Dollahite and R.D. Radeleff was designated to conduct the first examination leading to Diplomate status in the ABVT. Specific training and experience requirements were established for applicants and approval of each applicant's credentials was necessary before the candidate was admitted to the examination. Satisfactory completion of a comprehensive written examination was the final requirement for certification and the privilege of adding "DABVT" behind the

successful candidate's name. The first ABVT certifying examination was held in July 1967 in Dallas, Texas. The five successful candidates joined the three original members of the Certifying Board to form the initial group of certified, i.e., "Boarded," Veterinary Toxicologists (Oehme, 1970).

Since that time, annual certifying examinations of the ABVT have been given associated with the Annual Meeting of the AVMA. This certifying body has continued to set and maintain standards of qualification for veterinary toxicologists, and has complemented the continuing growth of veterinary toxicology experience and knowledge. By 2007 a total of 115 veterinarians have successfully completed the examination challenge and become Diplomates. Their special talents and skills continue to be professionally applied in academia, in industrial roles, as regulatory officials, at poison control centers and within diagnostic laboratories, and in consulting responsibilities throughout the world.

In the years since the discipline's early embryonic period, veterinary toxicology has evolved into a multidisciplinary focus that embraces all of the basic and clinical sciences. Its unique focus is not only the diversity of its embracing activities, but also the many talents and energies of its participants. It harbors a true global theme and is proud of its recognition and membership in "the only medical profession licensed for treating more than one animal species."

## PUBLISHED VETERINARY TOXICOLOGY LITERATURE

Individual authors in the past have attempted to summarize the concepts of veterinary toxicology. The most recent is *Veterinary Toxicology: Basic & Clinical Principles*; an encyclopedic documentation of the developments in veterinary toxicology over the past four decades with glimpses into the promises of exciting future growth. In logical and well-organized fashion, the contributors cover the vast and dynamic field of veterinary toxicology.

The tome on "General Principles of Veterinary Toxicology" is by one of the ABVT certified veterinarians from the initial 1967 examination in Dallas. The appropriateness of this contributor being a 40+-year Boarded veterinary toxicologist should not be lost to readers. The intensity and diversity of veterinary contributions to toxicology – pharmacokinetics, testing models, epidemiology, regulatory concerns, the timely heightened awareness of terrorism and the increasing necessity for legal compliance and actions – are documented.

Any toxicology text would be remiss if it did not focus on individual organ systems and their respective



toxicological effects and clinical manifestations. Sections move through each biological system and end with immunotoxicology and the disastrous effect that various chemicals can have by upsetting this balance of nature.

Of more recent origin are the veterinary efforts of exploring nanoparticles, radiation, and the mechanisms and models of investigative carcinogenesis utilizing various animal species. The veterinary toxicologist is foremost in working with such models and evaluating study results. Of additional current importance are the chapters on over-the-counter drug toxicity and the prevalent potential of various drugs of abuse to affect animal health.

The traditional group of toxic elements is intelligently and dramatically discussed in Section V, where metals and micronutrients ranging from aluminum through zinc are laid out in all their toxicity. No group of toxic elements is more historically relevant to toxicology than compounds such as arsenic, copper, fluoride, lead, mercury, selenium, and zinc, and when interspersed with some of the minor minerals, a complete array of metal and mineral animal intoxications is provided in this section.

The original emphasis for development of veterinary toxicology comes to the forefront in the middle of this volume. The organochlorines and the organophosphates/carbamates are extensively reviewed. Rotenone sneaks in, but the more recent toxic developments with pyrethroids, fipronil, imidacloprid, amitraz, and ivermectin and selamectin are prominently presented. The section on rodenticides and avicides, as well as the brief section on herbicides and fungicides, highlight the array of agricultural chemicals that have spurred not only the long-term developments in toxicology but also the environmental impact of widespread use of these groups of compounds.

The environmental areas of veterinary toxicology are discussed by reviewing industrial toxicants and the residual impacts of the biphenyls, dioxins and dibenzofurans. The environmental impact of these and other chemicals found in the environment are highlighted by extensive chapters dealing with their toxicity in birds, an introduction to ecotoxicology, and the distribution of chemicals in the global marine environment through aquatic toxicology, and the adverse effects of cyanobacterial toxins and others affecting marine animals. The extensive information on botulinum neurotoxin and the enterotoxins is not overlooked. Neither are the poisonous and venomous compounds generated by animals in the terrestrial environment. The chapter on "Mare reproductive loss syndrome" presents up-to-date information on this event's disastrous effect on equine breeding stables and the puzzling origin of these problems. An in-depth discussion on chemically induced estrogenicity and phytoestrogens brings readers current with this unique toxic hazard in all animal species including humans.

Section XIV is another expansive discussion of the still important poisonous plant concerns that contribute to

and continue to stimulate the interests and skills of veterinary toxicologists. The groups of important poisonous plants of the USA, Europe, Australia and New Zealand are reviewed, and then specific categories of plant toxins are presented: cyanide; nitrate/nitrite; oxalates; *Datura* and related plants; fescue; mushrooms; cottonseed toxicity; and the *Taxus* alkaloids. All these are common and highly concerning dietary risks for livestock and other animal species existing in the natural environment.

Fungal toxins are grouped under the "Mycotoxins" section where aflatoxins, trichothecenes, zearalenone, fumonisins, ochratoxins/citrinin, slaframine, ergot, and the interestingly and dynamic tremorgenic mycotoxins are nicely presented. These compounds present not only animal hazards, but are also important public health concerns for the dietary contamination of grains and other human food sources. Other dietary contaminants are reviewed in the section dealing with "Feed and water contaminants." Melamine and cyanuric acid, ionophores and nonprotein nitrogen dietary supplements are highlighted. Not to be overlooked, water quality and contaminants of water sources alert diagnosticians to the hazards and often animal-threatening risks involved with these aqueous contaminants.

The concluding sections in this book of facts and knowledge address how current methodology allows confirmation of specific poisonings and the appropriate means by which poisoned animals may be treated and managed. After reviewing the basic concepts of analyses, appropriate sample submission requirements for such procedures, the use of proteomics for diagnostic application, and the application of microscopic analyses of feeds and animal ingesta for toxic components in the diagnostic process are presented. To wrap it all up, a concluding section on therapeutic measures offers recommendations on how to prevent poisonings and, if necessary, what treatments may be applied to treat individual intoxications.

The basic principles of veterinary toxicology have been utilized to understand the mechanisms of toxicology, to relate to the numerous and challenging individual chemical constituents that offer risk and produce injury to animals and indirectly to humans, and to offer current information and recommendations for identifying such problems and specifically managing their animal and public health effects.

## ...AND WHERE WILL VETERINARY TOXICOLOGY GO FROM HERE?

It should be apparent that veterinary toxicology is about everything – from initial concerns of animal illness to specific molecular and genomic impacts in all of society.

The veterinary toxicologist is well equipped and active in identifying the opportunities and challenges presented. The discipline stands increasingly ready to contribute to medical science by utilizing its broad talents to have significant impacts for the health of all animals on this globe.

What's veterinary toxicology all about? The answers are to be found in this second edition and other volumes likely to be available in future years! May you use this forthcoming information and challenges to the benefit of society and science!

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# Concepts in veterinary toxicology

Roger O. McClellan

## INTRODUCTION

Toxicology, from the Greek words *toxicon* for poison and *logos* for scientific study, is the study of poisons. Veterinary medicine is that branch of medical science concerned with the diagnosis, treatment and prevention of diseases in animals. The adjective veterinary is derived from latin – *veterinae*, beasts of burden. Obviously, the modern field of veterinary medicine extends beyond the “beasts of burden” to include all the domesticated animal species, both livestock and companion animals, as well as non-domesticated species. Indeed, it has expanded to include non-mammalian species. While the focus of toxicology remains on chemicals, it is generally acknowledged that the study of effects of ionizing radiation is a part of the field or at least a closely related specialty. Pharmacology, from the Greek words *pharma* for drugs and *logos* for scientific study, is a closely related field concerned with the science of drugs: their preparation, properties, effects and uses in the diagnosis, treatment and prevention of disease.

The field of toxicology is very broad, including the identification and characterization of poisons, their physical and chemical properties, their fate in the body and their biological effects. In addition, toxicology is concerned with the treatment of disease conditions caused by poisons. The term toxicant and poison are used interchangeably. A toxicant is a material that when it contacts or enters the body via ingestion, inhalation, dermal contact, or injection interferes with the normal biological processes and causes adverse health effects. The term toxin is used to describe poisons originating from biological processes. The term toxic is used to describe the effects of a poison on biological systems.

Toxicosis is the term used to describe the syndrome of adverse health effects that result from exposure to a toxicant. During the last several decades, increased concern has developed for the effects of long-term low-level exposures to toxicants. With these exposures, adverse health effects, if they occur, may be manifest in a non-specific manner as an increase in the incidence of common diseases in a population.

A wide range of materials are capable of producing toxic effects when exposure occurs at sufficiently high levels. Indeed, with extreme levels of exposure most agents can produce adverse effects. For example, while both water and oxygen are required to sustain life they are toxic when the level of intake is excessive. The nature of the toxic responses depends not only on the toxicant but also the route of exposure, the duration and intensity of the exposure, and the characteristics of the exposed individual, i.e., species, gender, age, pre-existing disease states, nutritional status and prior exposure to the agent or related compounds. The exposure may be brief or prolonged. The response may occur acute or chronic and occur soon after exposure or much later and only after prolonged exposure. The response may be relatively unique to the toxicant, i.e., a specific toxicosis, or distinguishable from common diseases caused by natural processes or exposure to other agents. In many cases, sophisticated statistical methods are required to associate some excess health risk, over and above that caused by other factors, with a particular toxicant exposure. This is especially true today after much progress has been made in controlling exposure to toxic materials.

In this chapter, I first provide a brief historical perspective on the development of veterinary toxicology as a sub-specialty of the veterinary medical profession and



as a specialized area within the general field of toxicology. This is followed by a section on the evolution of veterinary toxicology from an observation-based profession and science to one that places increasing reliance on science developed through experimentation. This includes a discussion of the risk paradigm which has become an integral part of toxicology in recent decades. In the next section, I offer several related paradigms for acquiring, organizing and using knowledge in veterinary toxicology so as to maximize its potential impact. Next, there is a section on the sources of information that may be obtained either through observation or experimentation. These sources may include studies on the species of interest, i.e., people or some other specific animal species, controlled exposure studies in the species of interest, studies in other species, investigations using tissues and cells and structure–activity analyses. This is followed by a section discussing the design of experimental studies to optimize the interpretation and use of the results. The chapter concludes with a discussion of key toxicologic descriptors and a brief conclusions section.

## HISTORICAL PERSPECTIVE

### Historical events

The father of modern toxicology is generally acknowledged to be Aureolus Philippus Theophrastus Bombastus von Hohenheim (1493–1541), who referred to himself as a Paracelsus, from his belief that his work was beyond the work of Celsus, a first century Roman physician (Pagel, 1958). Paracelsus is credited with the well-known statement – “All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy.” Paracelsus advanced many views that were revolutionary for his time that are now accepted as fundamental concepts for the field of toxicology. In contrast to earlier emphasis on mixtures, he focused on the toxin as a specific primary chemical entity that was toxic. Paracelsus advanced four fundamental concepts:

- 1 Experimentation is required for examining responses to chemicals.
- 2 A distinction should be made between the therapeutic and toxic properties of chemicals.
- 3 The therapeutic and toxic properties are something closely related and distinguishable by dose.
- 4 It is possible to ascertain a degree of specificity for chemicals and their therapeutic or toxic effects.

It is obvious from the foregoing that toxicology and pharmacology are closely related fields of scientific endeavor. Pharmacology is focused on drugs, including

both their effectiveness and safety. Toxicology is concerned with all kinds of chemicals and other agents that may, at some level of exposure, cause adverse health effects. As will be noted at several places in this chapter, toxicology is increasingly concerned with low-level exposures for which the effects, if any are observed, may not be specific to a particular chemical.

Toxicology, in a sense, dates back to the earliest activities of humans. By observation, people came to learn that which could be ingested without harm and, by contrast, the foodstuffs to be avoided because of their harmful properties. They also came to know which animal venoms, plant extracts and other materials could be used for hunting, warfare and assassination. No doubt as animals were domesticated, it became apparent that the human observations and practices could be extended to domestic animals. Unfortunately, domestic animals are not always as astute as people in learning to avoid poisonous plants and other harmful situations. Thus, veterinary practitioners still encounter toxicoses involving animals ingesting poisonous plants.

The history of toxicology has been well documented by several contemporary authors (Milles, 1999; Gallo, 2008; Lane and Borzelleca, 2008). The history of veterinary toxicology has not been as well documented, although it is apparent that veterinary toxicology has been an integral part of veterinary medicine since the earliest time of the profession. Veterinary medicine is a specialized branch of medical science with formal programs of study leading to a professional degree. The history of veterinary medicine has been reviewed by several authors (Smithcors, 1957; Stalheim, 1994; Swabe, 1999; Wilkinson, 2005). The role of veterinary toxicology in the veterinary curriculum is well documented for one of the earliest veterinary medical colleges, that at the Free University of Berlin. Wilsdorf and Graf (1998) provide an account of the development of veterinary toxicology at that university from 1790 to 1945. Oehme (1970) has briefly reviewed the development of veterinary toxicology as a discipline in the United States.

### Textbooks

In the English language, the earliest veterinary toxicology publication I am aware of is a *Synopsis of Veterinary Materia Medica, Therapeutics and Toxicology* (Quitman, 1905) apparently used at Washington State University College of Veterinary Medicine in the early part of the 20th century. I am uncertain of the extent to which this synopsis may be based on a French text by Kaufmann (1901). The earliest English language veterinary toxicology textbook I was able to locate was that authored by an Englishman, Lander (1912). This book was also prepared in a second edition (1926) and a third edition

was prepared by an Irishman, [Nicholson \(1945\)](#). I am uncertain how widely it was used in the United States. The text included four sections; a brief introduction to toxicology followed by sections on classes of toxicants; mineral or inorganic poisons, organic poisons and drugs; and poisonous plants. This last section represented half of the book.

Many early students in veterinary medicine in the United States used textbooks prepared for physicians such as [Kobert \(1897\)](#), *Practical Toxicology for Physicians and Students*. It was also common to use either textbooks in pharmacology or veterinary pharmacology that contained a brief coverage of toxicology. Indeed, few veterinary medical colleges prior to the 1950s had full-time veterinary toxicologists on their faculty. Lectures on toxicology were usually included in courses in pharmacology, pathology and clinical medicine.

The first veterinary toxicology text I personally used was authored by [Garner \(1957\)](#) who was then a Senior Lecturer in Chemical Pathology (Veterinary) at the University of Bristol in the United Kingdom and later Head of the Radiobiology Department at the Agricultural Research Council Field Station, Compton, Berks, UK. The text by [Garner \(1957\)](#) was intended as a successor to the third edition of Lander's *Veterinary Toxicology* ([Nicholson, 1945](#)). A second edition was prepared by [Garner \(1961\)](#) after he became head of the Public Health Section, Radiological Protection Division, UK Atomic Energy Authority, Harwell, Berks, UK. Later, Garner came to the United States where he was initially associated with Colorado State University directing studies of the long-term effects of external radiation on beagle dogs. I recall asking Garner in the early 1970s about the possibility of preparation of a third edition of his *Veterinary Toxicology* text. He indicated that the field of veterinary toxicology had become so broad that it was not readily feasible for a single individual to author a text in veterinary toxicology and he was not interested in "shepherding" a herd of individual chapter authors with specialized knowledge of various aspects of veterinary toxicology.

[Radeleff \(1964\)](#) authored one of the first veterinary toxicology texts published in the United States. A second edition appeared in 1970. This was followed by a text prepared by [Osweiler et al. \(1985\)](#). Several books published in the 1960s became classics on the effects of poisonous plants ([Kingsbury, 1954, 1964](#); [Hulbert and Oehme, 1968](#)). Recent books on poisonous plants have been authored by [Garland and Barr \(1998\)](#), [Burrows and Tyrl \(2006\)](#) and [Knight and Walter \(2001\)](#). [Murphy \(1996\)](#) has authored a field guide to common animal poisons. It is organized by the organ system affected and then by toxicant.

[Osweiler \(1996\)](#) has authored a text focused on toxicology as part of the National Veterinary Medical Series

for Independent Study. It has been widely used by individuals preparing for the National Board Examinations for Veterinary Medical Licensing. [Roder \(2001\)](#) has prepared a text, *Veterinary Toxicology*, as part of a series *The Practical Veterinarian*. [Plumlee \(2004\)](#) has edited *Clinical Veterinary Toxicology* and [Peterson and Talcott \(2006\)](#) have edited two editions of *Small Animal Toxicology*. The present multi-authored text edited by Ramesh Gupta promises to be the most comprehensive text on veterinary toxicology published to date. It is encouraging that it is now appearing as a second edition. A veterinary toxicology text edited by [Chapman and Campbell \(2011\)](#) is in preparation.

There are a number of comprehensive general toxicology texts available today. I will note four that the serious student of toxicology will find useful to have in their reference library. *Casarett and Doull's Toxicology: The Basic Science of Poisons* edited most recently by [Klaassen \(2008\)](#) was first published in 1975 and is now in its seventh edition. As an aside, I had the opportunity to observe first hand Louis Casarett and John Doull planning the first edition of this new textbook as I served with them on the Toxicology Study Section of the National Institute of Health. Unfortunately, Louis Casarett died of cancer before the first edition ([Casarett and Doull, 1975](#)) was completed. I was pleased when John Doull asked me to prepare the chapter on Radiation Toxicology which my colleague, Charles Hobbs, and I did ([Hobbs and McClellan, 1975](#)) and which Louis intended to write. [Hayes \(2008\)](#), *Principles and Methods in Toxicology*, is now in its fifth edition. *Toxicology*, edited by [Marquardt et al. \(1999\)](#), built on an earlier German text by [Marquardt and Schafer \(1994, 2004\)](#). *Biological Concepts and Techniques in Toxicology: An Integrated Approach* edited by Riviere was released in 2006. Serious students will also want to be aware of a multi-volume comprehensive set of toxicology textbooks. The first edition was edited by [Sipes and colleagues \(1997\)](#) and a 14-volume second edition was edited by [McQueen \(2010\)](#). Moreover, there are numerous text and reference books available now covering various sub-specialty areas such as *Inhalation Toxicology*, *Reproductive and Developmental Toxicology* and *Dermal Toxicology*.

Some readers of this text will also be interested in the related field of environmental medicine which has largely developed with a focus on the influence of the environment on human health. Without question, there is a need to integrate veterinary medicine and specifically veterinary toxicology into environmental medicine. A recently released text, *Environmental Medicine* ([Ayres et al., 2010a](#)), includes a lead chapter that details the context for environmental medicine ([Ayres et al., 2010b](#)). That chapter reviews important information on causes of death in low- and middle-income versus high-income populations ([Lopez et al., 2006](#)) and the

role of environmental versus non-environmental factors as contributors to the global burden of disease (Kung *et al.*, 2008). Recognizing that this text edited by Gupta is intended for international readers, the lead chapter in Ayres *et al.* (2010b) serves as a reminder of the wide range of environmental conditions experienced by people around the world. Veterinarians have an important role to play in promoting human health around the world in multiple ways such as by facilitating the production and availability of safe food products of animal origin and aiding in control of infectious agents.

In addition to text and reference books, there are numerous journals published in the field of toxicology that regularly contain articles that relate recent findings in veterinary toxicology. Many clinically oriented veterinary medical journals contain articles on veterinary toxicology. The online search capabilities serving the medical sciences, including toxicology and veterinary toxicology, are expanding at an exponential rate. Of special note are those maintained under the auspices of the National Library of Medicine, MEDLINE and TOXLINE.

## Organizations

A number of professional scientific organizations have been created as the field of toxicology, including veterinary toxicology, has matured. The most noteworthy include the American College of Veterinary Toxicology (ACVT), American Board of Veterinary Toxicology (ABVT), Society of Toxicology (SOT), American Board of Toxicology (ABT), and Academy of Toxicological Sciences (ATS). The ACVT was one of the earliest scientific societies in the field, being founded in 1958. It now exists as the American Academy of Veterinary and Comparative Toxicology. The ACVT was instrumental in fostering the creation of the ABVT and its recognition by the American Veterinary Medical Association (AVMA) as the approved certifying specialty organization for veterinary toxicology. Three well-known veterinary toxicologists, W. Binns, J.W. Dollahite and R. Radeleff, were accepted by the AVMA as Charter Members of the ABVT. They prepared the first certifying ABVT examination which was given in 1967 (see [www.abvt.org](http://www.abvt.org)). I was pleased to be one of six individuals in the first class certified, based on examination, as Diplomates of the ABVT.

The SOT, with the world's largest membership of toxicologists, was organized in 1961 (see [www.sot.org](http://www.sot.org)). Many of the organizers of the SOT were members of the American Society for Pharmacology and Experimental Therapeutics (ASPET) who felt toxicologists needed a "home" of their own. I recall attending an organizational meeting of the SOT held in conjunction with an ASPET meeting at the University of Rochester and the excitement

and enthusiasm of the attendees for creating the SOT. As an aside, it would be a few years before I felt my credentials were sufficient that I could apply for membership in the SOT. The SOT includes a number of specialty sections including the Comparative and Veterinary Specialty Section and Toxicologic and Exploratory Pathology Specialty Section with veterinarians being a majority of the membership of these sections. Veterinarians have played a major role in the SOT from its founding with many serving in leadership roles. Four veterinarians (Perry Gehring, Frederick Oehme, Roger McClellan and James Popp) have served as president of the SOT.

The SOT fostered the creation of the ABT which held its first certifying examination in 1980 (see [www.abtox.org](http://www.abtox.org)). I was pleased to be one of the first class of individuals certified, based on examination, as Diplomates of the ABT.

A third certifying entity, the ATS, which accepts individuals as fellows based on a review of credentials, was created in 1981 (see [www.acadtoxsci.org](http://www.acadtoxsci.org)). Many veterinary toxicologists belong to all of the organizations noted above and some have been certified by one or more of the certifying organizations; the ABVT, ABT and ATS. Of special note, four veterinary toxicologists (Roger McClellan, Charles Capen, Jim Riviere and Bernard Schwetz) have been recognized for their contributions to human health by election to the Institutes of Medicine of the National Academies of Sciences. Veterinary toxicology has made major contributions as it has continued to evolve over the past decades along with the general field of toxicology.

## EVOLUTION OF VETERINARY TOXICOLOGY

### Roots in veterinary medicine and toxicology

The evolution of veterinary toxicology occurred concurrently with evolution of its two roots; the profession of veterinary medicine and the science of toxicology. The veterinary medicine profession was initially focused on domestic animals, particularly those used for food, fiber, transportation and to provide power for agricultural endeavors and transportation. With the growth of more specialized agriculture and production practices, the profession with its linkage to domestic livestock, stimulated growth of veterinary toxicology. Veterinary toxicology in its earliest years had a major focus on poisonous plants and then on antidotes for various toxins. The early part of the 20th century presented a special challenge for veterinary medicine as the use of horses and mules in agriculture decreased in favor of the use of equipment powered by internal combustion engines. During this period of time, there must have been

considerable uncertainty as to the future of the veterinary medical profession.

By the mid-20th century three movements transformed veterinary medicine. The first related to the traditional roots of the profession in animal agriculture and related to the increasing emphasis given to large-scale highly specialized domestic livestock endeavors. The second related to the increased attention given to providing veterinary medical services to a growing population of companion animals including horses, dogs and cats. In both areas the science of veterinary medicine was strengthened as observation-based medical practice was complemented and, ultimately, supplemented by science-based medicine. During this period, veterinary toxicologists began to play an important role in veterinary medical diagnostic laboratories, both in veterinary medical colleges and in state and federal agencies. With the strengthening of the science base of veterinary medicine, including the quality of the science in the veterinary medical curriculum, the third movement, the emergence of the comparative medicine character of veterinary medicine became more apparent and was enhanced (Wilkinson, 2005). These changes in the profession were accompanied by increased involvement of veterinarians in research on the species of traditional concern to the profession, domestic and companion animals (Stahlheim, 1994), and to participation in a broader range of biomedical research activities, involving use of the traditional laboratory animal species, driven largely by concern for human health (Wilkinson, 2005).

### Emergence of science-based toxicology

Toxicology, like veterinary medicine, was also rapidly changing and evolving in the mid-20th century. The previous strong emphasis on field observations was first complemented and then supplemented by experimentation. This, in turn, led to the current strong mechanistic orientation of toxicology. With this shift in toxicology came an increased awareness of the utility of a comparative medicine orientation in research directed primarily toward improving human health (Wilkinson, 2005). With this comparative medicine orientation came increased opportunities for individuals educated in veterinary medicine, including veterinary toxicology, to contribute to general toxicology and biomedical science.

These changes in the veterinary medical profession and the emergence of toxicology as a science came during a period when the public was giving increased attention to the health risks, and its counterpoint-safety, of new technologies and products. World War II resulted in the creation of the Manhattan Project in the U.S. to develop nuclear weapons. Following the war, the Manhattan Project became the Atomic Energy

Commission (AEC) with a broad mission that included peaceful applications of nuclear energy. A series of national and specialized laboratories were created with several having a mandate to understand the effects of external radiation and internally deposited radionuclide. Veterinarians had a key role in many of these laboratories. My career began at one of the major AEC laboratories, the Hanford Laboratories in Richland, WA, where research was conducted on a range of topics including effects of radioactive materials on domestic animals, a research program initially led by the late Leo K. Bustad, who later served as Dean of the College of Veterinary Medicine at Washington State University. The Hanford Laboratories continue today as the Pacific Northwest Laboratories operated by Battelle Memorial Institute. Later I would lead one of the specialized AEC laboratories, the Lovelace Inhalation Toxicology Research Institute (ITRI), which continues today as part of the Lovelace Respiratory Research Institute. The mission of ITRI was to study the health consequences of exposure to radioactive materials and, then later, a broad range of airborne materials including vehicle emissions. The research conducted at Hanford, Lovelace ITRI and in numerous other laboratories with support from the Atomic Energy Commission, which later became the Energy Research and Development Administration, and then soon became the Department of Energy, provided an enormous knowledge base on the health effects of exposure to both external radiation and radionuclides that would be deposited internally. Contrary to the impression gained from many popular media reports, our understanding of the health effects of radiation probably exceeds our knowledge of any other toxicant.

The post-World War II era was also a period of substantial growth of the chemical and pharmaceutical industries, especially in the U.S. and in Europe. This included development of a wide array of new products including many chemicals used in agriculture and from the pharmaceutical industry the development of many new drugs including many widely used in veterinary medicine. The growth of these industries was intertwined with an increase in research and development. This included substantial research to ensure the safe manufacture of products and their safe use. This continued a tradition begun pre-World War II when the major chemical companies such as Dupont, Dow, Union Carbide, Eastman Kodak and others created laboratories that had a strong toxicology orientation. Many veterinarians played a key role in these laboratories. Unfortunately, these laboratories today are much smaller or have even disappeared.

Despite the substantial knowledge base that was being created and used to guide the safe use of chemicals concerns developed. This concern was the focus of Rachael Carson's book *Silent Spring* (Carson, 1962). She



focused on both human health impacts and impacts on the total ecosystem of which people were just a part. Her book was certainly one of the key stimuli to a tidal wave of legislative actions in the United States that focused broadly on the environment with concern for clean air and water; safe food, pharmaceuticals, pesticides, fungicides, rodenticides, and consumer products; and a safe working environment.

The legislative actions and related administrative actions in the 1970s created the U.S. Environmental Protection Agency, the Consumer Product Safety Commission, the National Institute for Occupational Safety and Health, the National Center for Toxicological Research, the National Institute of Environmental Health Sciences and the Cancer Bioassay Program within the National Cancer Institute, which evolved into the National Toxicology Program now administered by the National Institute for Environmental Health Sciences. This was also a period of rapid expansion of research activities in the pharmaceutical, food, chemical and petroleum industries. The major chemical companies working together started in 1976 the not-for-profit Chemical Industry Institute of Toxicology, which now exists as the Hamner Institutes for Health Sciences, to test commodity chemicals, investigate the mechanisms of chemical toxicity and train additional toxicologists. The Food and Drug Administration continued its traditional dual emphasis of ensuring both the efficacy and the safety of drugs and medical devices continued. Increased emphasis was given by the FDA to veterinary drugs and to the potential for veterinary drugs to contaminate meat and milk.

Increasing public concern for safety/risk and the resulting legislation led to the development of increasingly formalized approaches to both safety and risk analysis. This included more clearly defined roles for using the results of toxicological studies, including studies with laboratory animals, to assess the safety, or conversely risk, to humans of the use of pharmaceuticals, other products in commerce, and technologies.

### Toxicology joined to the risk paradigm

As noted earlier, federal legislation passed in the 1970s focused on health impacts of environmental and occupational exposures and led to more formalized approaches to evaluating the risks and safety of various exposures. The risk paradigm built on the long-standing paradigm linking sources to exposure to dose to adverse health outcomes that had guided toxicology from its earliest days (Figure 2.1). I have reviewed elsewhere the development of the risk analysis paradigm (McClellan, 1999, 2010). The development of risk analysis as a specialized area of interdisciplinary science led to the founding of

the Society for Risk Analysis in 1981. The risk analysis paradigm originally proposed by the National Research Council (1983) and used by the USEPA is shown in Figure 2.2. A later report "Science and Judgment in Risk Assessment" (NRC, 1994; McClellan, 1994) and reports from the Risk Commission (1997) reaffirmed use of the risk paradigm which continues to be a cornerstone of activities not just at EPA but in other national and international agencies and in the private sector.

The original key elements of the risk paradigm were: (1) hazard identification, (2) exposure-response assessment, (3) exposure assessment, and (4) risk assessment. The NRC (1994) report emphasized the importance of a fifth element – using the results of the risk analysis to guide future research and, thus, reduce uncertainty in future risk estimates. In addition, I have added a sixth overarching element – risk communication. The hazard identification element has been a source of contention and confusion both with the public and in the scientific community, especially with regard to cancer as I will discuss later.

Hazard is defined as the potential for an agent under some conditions of exposure to cause an adverse effect (NRC, 1983, 1994; McClellan, 1999, 2010). With this definition the level of exposure or dose required to produce an adverse health effect is not considered. An agent may be classified as a hazard irrespective of whether or not the exposure conditions required to elicit adverse effects under experimental conditions are relevant to human situations. The exposure-response assessment involves characterization of this relationship as it may pertain to likely levels of human exposure. The exposure assessment quantifies, either retrospectively or prospectively, the likely duration and intensity of human exposure to the hazardous agent. The risk assessment element brings together information from the other three elements to characterize risk as illustrated in Figure 2.1. Risk is defined as the probability of occurrence of an adverse health effect from exposure to a hazardous agent at a specified duration and intensity of exposure. As an aside, especially in Europe, the word hazard is used as risk has been defined in the United States. Safety is defined as being a condition with a high probability of freedom from any increase in adverse health outcome when the agent is used in a specified manner. Obviously, both safety and risk are relative recognizing that it is not possible to ensure absolute freedom from some small level of risk. As the control of hazards and risks has improved, in part through more and more stringent regulations, scientists and society at large are increasingly faced with the challenge of how low is low enough?

The more formalized risk analysis approaches developed, starting in the 1970s, built on approaches developed earlier for providing guidance for controlling occupational exposures, the intake of contaminants

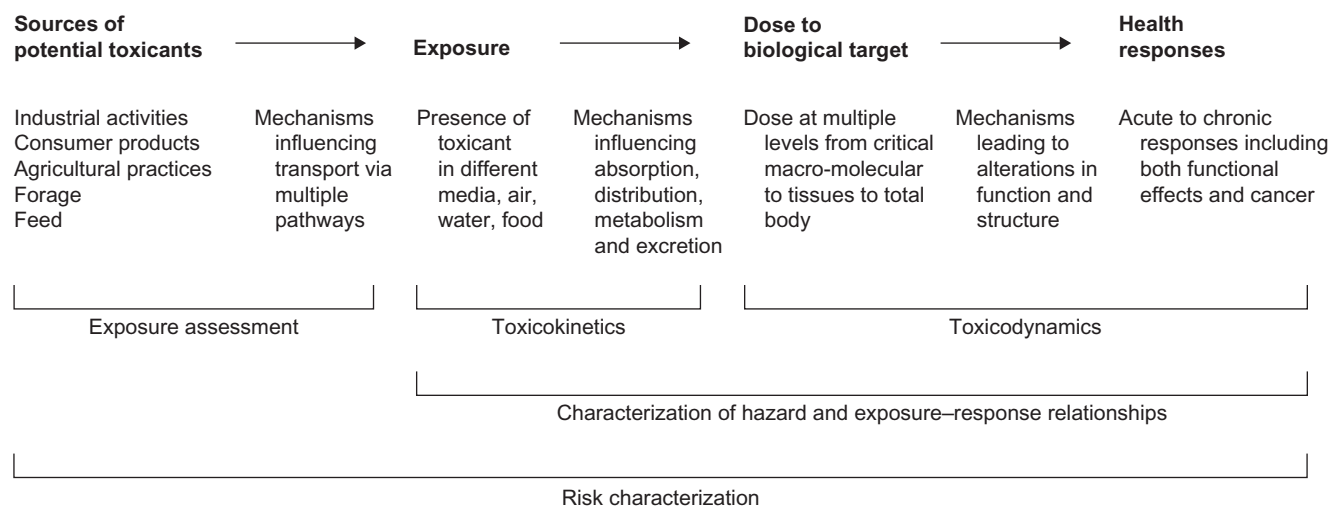


FIGURE 2.1 Critical linkages for integrating information from sources of toxicants to the development of adverse health effects.

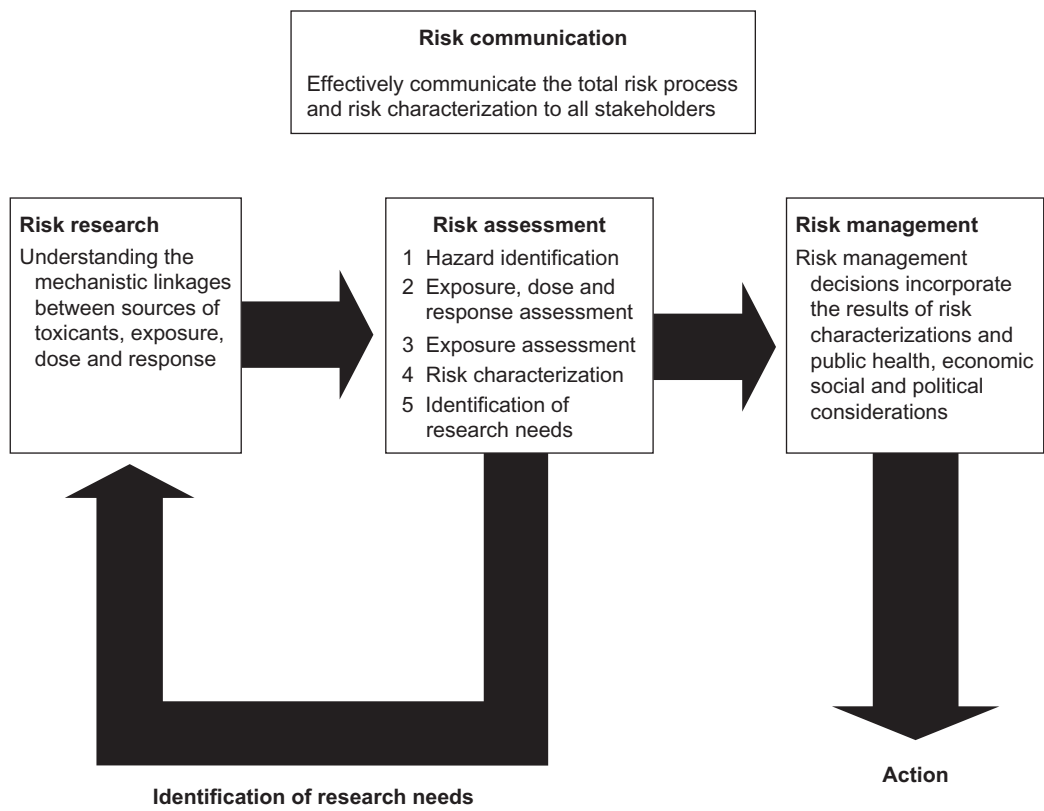


FIGURE 2.2 Risk paradigm for evaluating potential health impacts of a toxicant.

in food and the safety of pharmaceutical agents. Pre-World War II, the primary focus was on adverse health outcomes that caused functional impairment such as decreased respiratory function. As will be discussed later, the issue of carcinogenic responses received limited attention pre-World War II. The approach to developing guidance for the control of toxicants was based

on the assumption that a threshold exists in the exposure (dose)–response relationship – just as discussed by Paracelsus. The threshold exposure–response relationship is shown in Figure 2.3 along with four other relationships; sub-linear, linear, supralinear and a U-shaped or hormetic function. Note that both scales in this schematic rendering are logarithmic.

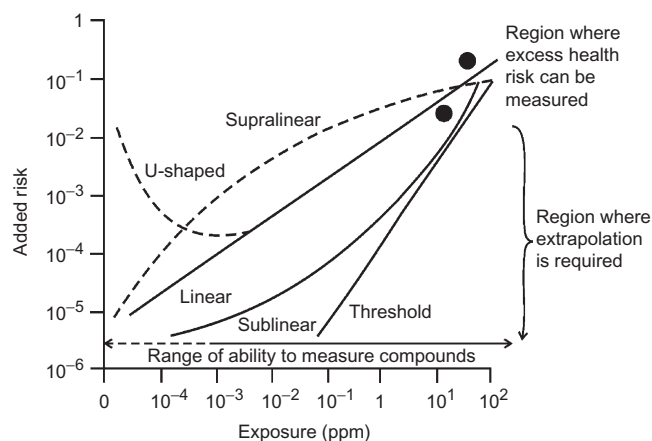


FIGURE 2.3 Schematic rendering of exposure-response relationships for various toxicants.

Technically, in hormesis there is a beneficial effect at some low level of exposure which decreases with increasing exposure/dose and at yet higher levels adverse effects become apparent. During the last decade, there has been increased discussion of the concept of hormesis in which very low level exposures have positive effects with negative effects observed only at higher exposure levels (Calabrese and Baldwin, 2003; Calabrese and Blain, 2005; Calabrese *et al.*, 2007). The concept of hormesis is well known to veterinarians who are aware that certain agents, such as vitamins and minerals, are essential for life at low concentrations and can produce toxicity with excess intake.

As an aside, there has been ongoing debate for decades as to whether linear exposure-response relationships, especially for cancer, are realistic, i.e., an added level of exposure, regardless of how small, results in a calculable monotonic increase in cancer risk. It has been argued by some that the linear exposure-response model is appropriate for regulatory purposes for assessing cancer risks because every dose of a new agent is added to a background of genetic damage in somatic cells arising from multiple agents and endogenous factors.

In recent years the debate over the nature of the exposure-response relationship has been extended from cancer as an endpoint to non-cancer endpoints. Arguments for the use of linear non-threshold exposure-response relationships for non-cancer endpoints have been advanced by White *et al.* (2009). An alternative view has been advanced by Rhomberg *et al.* (2011a), namely, that linear low-dose extrapolation for non-cancer health effects is the exception, not the rule. Extension of the debate over the nature of the exposure-response relationship to non-cancer endpoints, such as respiratory or cardiovascular morbidity and mortality, has important implications for assessing risks such as from air pollutants like particulate matter and ozone. For

example, the Clean Air Act (CAA, 1970) requires the EPA Administrator to set National Ambient Air Quality Standards (NAAQS) for these pollutants at levels that are protective of public health with an adequate margin of safety. If the exposure-response relationship for the pollutant in question has a threshold then the setting of the standard is relatively straightforward, identify the threshold concentration and set the NAAQS at a lower concentration for a given averaging time and statistical form. If a linear, no threshold, exposure-response relationship is apparent, the question arises as how to determine when the NAAQS is set low enough. McClellan (2011) has addressed that topic in a recent paper drawing on the guidance of Supreme Court Justice Stephen Breyer in the legal case of *Whitman v. American Trucking Associations* (2001). McClellan has emphasized the importance of “distinguishing between the science that informs the setting of the standard and the policy judgments inherent in selecting the standard”.

The early development of threshold limit values (TLVs) for control of occupational exposures by the American Conference of Governmental Industrial Hygienists, organized in 1938, assumed the existence of thresholds in exposure-response relationships. The initial data used in establishing TLVs were provided primarily by opportunistic studies of occupationally exposed human populations. In the absence of human data, data from controlled exposure studies in laboratory animals were used. This necessitated the use of safety factors to account for inter-individual variability, inter-species extrapolation, and duration of the study as will be discussed later. The original safety factors were formally proposed by Lehman and Fitzhugh (1954) of the Food and Drug Administration. In 1970, the USEPA was organized and began using the same factors. However, the EPA identifies them as uncertainty factors apparently out of a desire to avoid use of the potentially contentious word – safety.

Post-World War II increased public concern developed for the occurrence of cancer. This was stimulated by multiple factors. Extensive research conducted during and after the war on the effects of both external ionizing radiation and internally deposited radionuclides emphasized the importance of cancer as a radiation-induced disease. Concern for radiation-induced cancer was further heightened when the intensive follow-up of Japanese A-bomb survivors revealed an increase first in hematopoietic neoplasms and later in solid cancers. These findings soon led to abandoning a threshold approach to evaluating radiation risks in favor of using a probabilistic approach to assess the health risks of using radiation devices in space and nuclear power. The probabilistic approach using the linear exposure-response model discussed earlier was convenient to use because it could be readily applied to assessing the

risks to individuals or populations. My first experience with probabilistic risk assessment came in 1965 when I was on a temporary assignment with what was then the U.S. Atomic Energy Commission (AEC). I worked with a joint AEC–National Aeronautics and Space Administration Committee assessing potential human cancer risks of accidents involved with the launch of space craft containing plutonium-238-fueled thermal electric power systems.

Another factor influencing public concern was the increasing incidence of total cancers being observed in all of the economically developed countries including the U.S. driven largely by lung cancer. It is now well known that the increase in lung cancer, first observed in men and later in women, was largely related to cigarette smoking. Rachael Carson's book also created concern for exposure to man-made chemicals contributing to the increasing incidence of cancer and encouraged the view that somehow man-made chemicals were different than chemicals of natural origin. It is now known that this is not the case (Gold *et al.*, 2003). Ironically, some major commodity chemicals such as formaldehyde are normal constituents of mammals.

The experience with radiation soon resulted in its use as a proto-typical carcinogen in developing approaches to risk analysis and risk regulation. Albert (1994) documented the development of the USEPA's approach to assessing cancer risks. Key assumptions in the approach were: (1) cancer-causing chemical agents acted like radiation in causing cancer; (2) there was a linear relationship between exposure (dose) and increased risk of cancer extending to the lowest levels of exposure; (3) agents causing cancer in laboratory animals could be viewed as also causing cancer in people; and (4) exposure–response relationships could be extrapolated between species by considering differences, body weight and surface area, i.e., metabolic activity. These assumptions were viewed as default options to be used in the absence of specific scientific data to the contrary (McClellan, 1994, 1999, 2003; NRC, 1994).

In response to public concern for chemicals causing cancer, the International Agency for Research on Cancer (IARC), a part of the World Health Organization, was organized in Lyon, France, under the leadership of John Higginson to develop an international research program to study cancer. Higginson's deputy, Lorenzo Tomatis, provided leadership for creating a program to classify agents or occupations as to their carcinogenic potential with the results published in a series of monographs (IARC, 1972). The view was that if cancer-causing chemicals or other agents, such as radiation, or workplace conditions involving exposure to chemicals or other agents causing cancer could be identified, then these could be controlled and the occurrence of cancer in people reduced. The IARC carcinogen classification

scheme considers human, laboratory animal and supporting data to classify agents or workplace conditions as (1) carcinogenic to humans, (2) probably carcinogenic to humans, (3) possibly carcinogenic to humans, (4) not likely to be carcinogenic to humans, or (5) not classified as carcinogenic. The IARC classification is strictly hazard oriented; it does not formally evaluate the potency of these agents for causing cancer at a specific level of exposure.

The USEPA, the National Toxicology Program (NTP) and other organizations have developed similar carcinogen classification schemes (EPA, 1986, 2005a,b; NTP, 2005). In recent years, IARC (1991) has made provision for increased use of mechanistic data in classifying chemicals as human carcinogens. Both the EPA and NTP now also give increased emphasis to the use of mechanistic data in classifying chemicals as carcinogens (EPA, 2005a,b) unlike IARC and the NTP, the EPA does develop estimates of cancer causing potency for some agents classified as having cancer-causing potential. This in turn, using measurements or estimates of exposure, provides the basis for calculating lifetime cancer risks for individuals or populations.

It should be apparent that the classification of a given chemical or agent as to its cancer causing potential is insufficient for characterizing cancer risk since the hazard-based classification does not include an estimate of the agent's potency. The USEPA has estimated the carcinogenic potency for a number of chemicals. The results are usually related as the concentration of a chemical in water or air that will result in a calculated one in a million probability of cancer occurring above the background incidence (EPA/IRIS, 2011). In viewing such estimates it is useful to recall that in developed countries of the world with long-lived populations, about one third of the population will be diagnosed with cancer during their lifetime and one fourth will die with a cancer. To estimate the cancer risk for any agent and exposure situation, it is also necessary to estimate the exposure to the agent, both as to intensity, i.e., concentration, and duration. In short, risk is a product of exposure and the potency of the agent for causing the effect.

There has been a tendency for regulatory agencies, such as the USEPA, to use their experience with classifying chemicals as to their carcinogenic potential as a template for also classifying chemicals as to their potential for producing other non-cancer hazards. Thus, there has been a trend toward classifying chemicals as to their potential hazard for causing different health outcomes and labeling them as such, i.e., neurotoxins, reproductive toxins, hepatic toxins, etc. Indeed, some even broader classifications have emerged, i.e., endocrine disrupting chemicals. In my view, this short-hand approach to identifying and classifying hazardous agents as to their potential to cause cancer or other effects is often



confusing to the public. In my opinion, the labeling approach has contributed to both radiation phobia and chemical phobia and sometimes irrational actions. It certainly flies in the face of the fact that for many chemicals the admonishment of Paracelsus that “the dose makes the poison” remains true for many chemicals. For numerous chemicals, even when toxic effects are apparent at high doses, these same adverse effects are no longer manifest at sufficiently low doses. Gold *et al.* (2003) has discussed the challenge of using high-exposure (dose) animal studies to identify either man-made or natural chemicals as human carcinogens.

In recent years increasing attention has been given to developing safer products and technologies, especially when concerns have been raised with regard to the hazards of an existing product or technology. In some cases, the focus has been on developing products that do not have carcinogenic properties to replace products that have been classified as human carcinogens or probable or possible human carcinogens by IARC or as known or reasonably anticipated to be human carcinogens in the Report on Carcinogens prepared by the National Toxicology Program. Endpoints other than cancer have increasingly drawn attention for chemicals such as bisphenol A which has been under attack as an endocrine disrupting chemical. In general, chemicals that persist in the environment and have a long residence time in animals and humans, even when shown to have low hazard potential, have become targets for replacement. In yet other cases, the approach has been to identify replacements for chemicals that are persistent in the environment and have a long residence time in animals and humans. In other cases, the approach has been to find ways to minimize the use of the chemical viewed as being unsafe. These activities have given rise to a field of science sometimes referred to as “green chemistry” (Anastas and Warner, 1998).

Controversy has recently developed over cancer classification for new technologies or products with arguments developing over whether they are really safer. One example relates to diesel engine technology. Controversy continues over whether the particulate emissions from old diesel engines using high sulfur content fuels are or are not carcinogenic to humans (Hesterberg *et al.*, 2005, 2011). Much attention has been given to developing low emission, “green,” engines. The human epidemiological evidence for traditional diesel exhaust being carcinogenic based on epidemiological studies of occupational-exposed populations has been inconsistent. Long-term studies of rodents exposed to high concentrations of traditional diesel exhaust have been negative in Syrian hamsters and mice and positive in rats. The positive findings in rats have been attributed to an “overload phenomenon” and not been viewed as irrelevant to assessing human carcinogenic hazard.

Nonetheless, major effort has been given to improve reducing emissions by improving engine design, using ultra-low sulfur fuel, introducing exhaust after-treatment systems and using electronic control systems. Exhaust emissions from new technology diesel engines operating with ultra-low sulfur fuel (less than 15 ppm sulfur) of particulate matter and most other constituents are less than 1% of that observed for traditional diesel units (Hesterberg *et al.*, 2011). In my view, the emissions are so low they should be considered as “not carcinogenic to humans.”

Another new set of green products are reformulated synthetic glass fiber insulation products purposefully designed and produced to be “biosoluble” and lacking in carcinogenic properties (Hesterberg and Hart, 2001; Bernstein, 2007). The IARC (2002) previously determined that while it was appropriate to continue classifying some durable synthetic vitreous fibers as “possible human carcinogens,” it was not appropriate to classify “biosoluble” fibers with regard to human carcinogenic potential. The National Toxicology Program is currently evaluating how these new biosoluble glass fibers should be listed in the next Report on Carcinogens. Previous Reports on Carcinogens have treated synthetic vitreous fibers as a single category and classified the category as “reasonably anticipated to be human carcinogens.” An expert panel has unanimously recommended that the biosoluble glass fibers not be listed as “reasonably anticipated to be human carcinogens” while biodurable synthetic glass fibers continue to be listed as “reasonably anticipated to be human carcinogens.” It will be interesting to see how the NTP staff heeds this expert advice in formulating a final decision to list or not list biosoluble glass fibers as contrasted with durable synthetic fibers.

The outcome of the classification decisions for the new “green” technology (diesel engines with exhaust after-treatment devices and using ultra-low sulfur fuels) and green products (biosoluble glass fibers) have the potential for being precedent setting. If new “green” products/technologies cannot be accorded different cancer classifications than the products they replace, the public will be sent a confusing signal and industry may be reluctant to invest in developing new safer products. Conversely, recognition that the new products/technologies are safer may stimulate research and development on “green” replacement products and associated safety evaluation activities.

The situation in periodically re-evaluating the carcinogenic classification of a specific chemical is different than the re-evaluation of a technology or product. As noted for diesel engine technology and glass insulating fibers, they may change over time. Conversely, a specific chemical is always that chemical; however, the amount of information on the chemical will increase over time. Even with an increased body of knowledge, controversy

may continue. An example is formaldehyde which is both a normal constituent in the body as part of the 1-carbon metabolic scheme and also an important commodity chemical. A key issue in the controversy is the interpretation of weak epidemiological evidence of an association between inhalation exposure to formaldehyde and the occurrence of leukemia. Rhomberg *et al.* (2011) and Golden (2011) have critically reviewed the literature and emphasized the lack of biological plausibility for inhaled formaldehyde causing extra-pulmonary effects such as leukemia. A Committee of the National Research Council (NRC, 2011) has recently reviewed the Environmental Protection Agency's draft IRIS assessment of formaldehyde and addressed these issues.

Increasingly, the "precautionary principle" has dominated approaches to regulation of products and technologies. The concept took hold first within Europe and was codified by the European Commission (2002); more recently its use has been advocated in the United States. In its most simple form it argues that if the product or technology has not or cannot be viewed as safe then the product or technology should be replaced or not used. It is obvious that the "devil is in the details" with regard to the basis for characterizing what is safe or not safe. There is a continuum from safe products or technologies to highly hazardous products and technologies. Indeed, some highly hazardous products may be used in a controlled manner so they do not pose a significant health risk. Some advocates of the "precautionary principle" point to thousands of untested chemicals and argue they should be tested or banned. I would argue that a critical review of past experience with many of these chemicals or closely related chemicals provides a sound basis for their continued use. Quite frankly, additional extensive testing using laboratory animals may not be warranted.

The "precautionary principle" is a core element of the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) program established by the European Commission (2006). More details are available at REACH (2011). Thoughtful reviews of the REACH program have been written by deAvila and Sandberg (2006) and Williams *et al.* (2009).

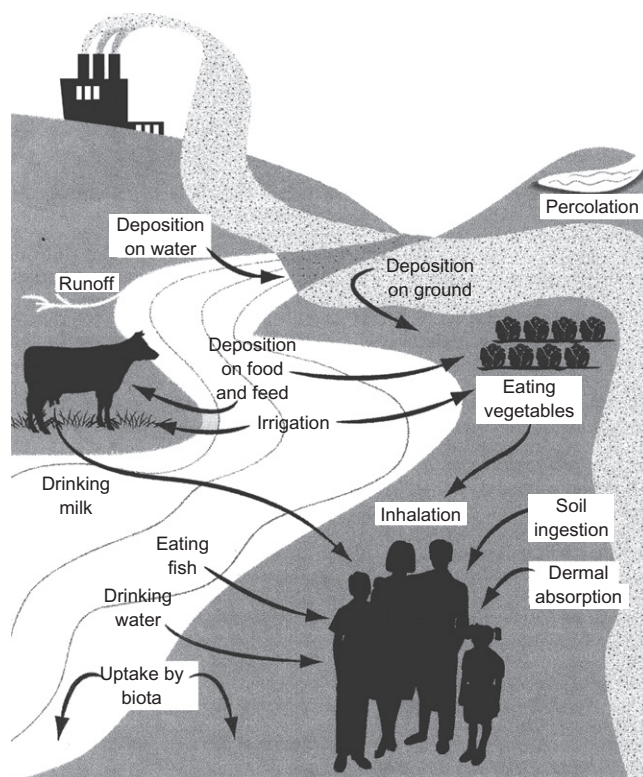
## A FRAMEWORK FOR ACQUIRING INFORMATION

### Linkages from sources to health impacts

The purpose of this section is to provide a conceptual framework for using information to evaluate specific cases of actual or alleged toxicosis and to facilitate the acquisition of new knowledge that will have impact in understanding potential toxic effects. Earlier, in Figure 2.1, a conceptual

framework was provided for evaluating the linkages extending from a source of a toxic material to manifestation of an adverse health outcome in an individual or a population. The conceptual framework is equally applicable to humans or other animal species.

The source to exposure linkage has been expanded in Figure 2.4 (Paustenbach, 2001). In this example, an industrial plant is illustrated as the source. The figure serves to illustrate the complex nature of the exposure pathways that may be encountered including the role of livestock. The focus in the figure is on the multiple pathways by which a potential toxicant may reach a human population; inhalation, drinking water, dermal absorption, ingestion of soil, and ingestion of a variety of food-stuffs including milk and meat from domestic animals. All of these pathways might also serve to expose the cow in the figure to the toxicant. Obviously, the quantities of the toxicant taken in by the single cow could also cause toxicity in a herd of cows. Equally as important is the role of the cow as a pathway for the toxicant to reach people. For example, the figure illustrates that a toxicant could be present in cow's milk and the milk could be consumed by people. The cow could also be slaughtered and the meat ultimately consumed by people. Thus, it is



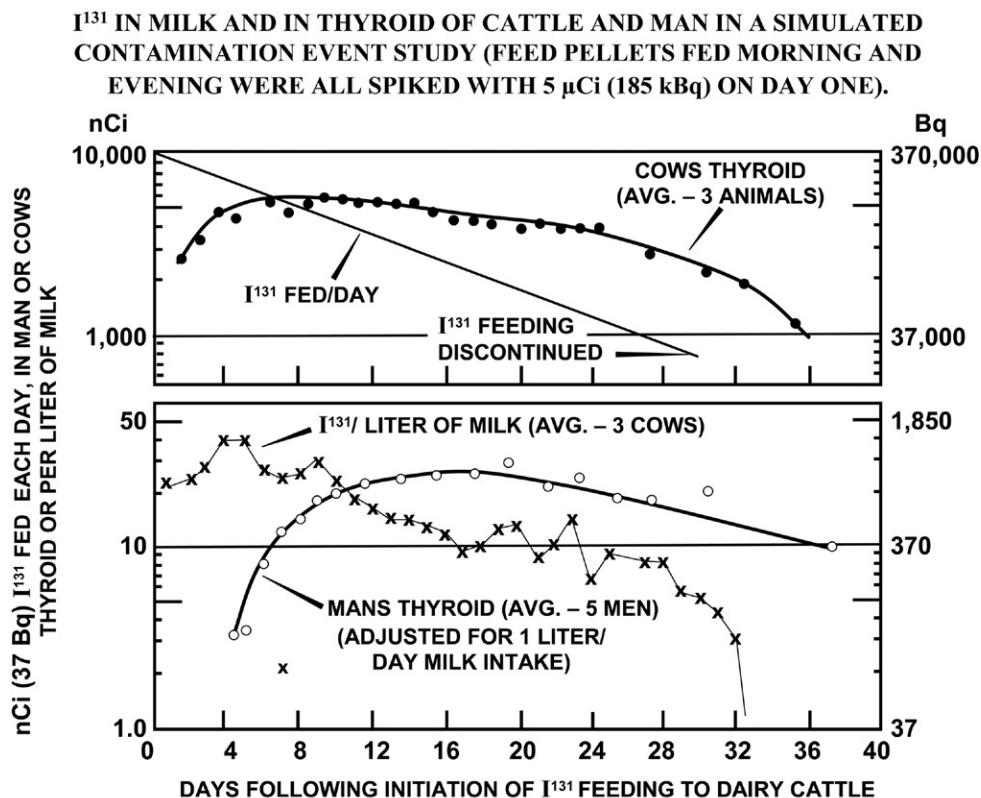
**FIGURE 2.4** Schematic rendering illustrating exposure pathways extending from a source of toxicants to exposure of livestock and people (from Paustenbach, 2001).

important to recognize that the cow, or any other food animal species, can both manifest toxic effects and serve as a pathway for toxicants to reach people via the food supply.

It is readily apparent that the schematic rendering shown in Figure 2.4 can be expanded or contracted. In natural ecosystems, multiple species might be involved as a toxicant moves from a source or multiple sources via various pathways. In some cases, various species in the ecosystem may be impacted as individuals. Moreover, natural populations may be impacted. In addition, these pathways may ultimately result in the toxicant reaching people. An example is mercury in fish. In practice, veterinarians may encounter situations where poisonous plants in the pasture or in harvested forage may be the source of the toxicant. Feed may be contaminated at a mill and serve as the pathway by which a toxicant reaches the livestock. In other cases, the potential human toxicant may be a pharmaceutical purposefully given to the cow.

The utility of using the conceptual approach in Figure 2.4 can be illustrated using the transfer of a specific radionuclide,  $I^{131}$ , from the diet of cows to the cow's

milk to intake by humans and thyroid uptake. This situation was of concern when nuclear weapons were being conducted in the atmosphere resulting in worldwide fallout and for releases from reactor accidents in England (Windscale), Ukraine (Chernobyl) and Japan (Fukushima). Figure 2.5 illustrates the results of a controlled study conducted at the Hanford Laboratories, Richland, WA, in the early 1960s with  $I^{131}$  administered to dairy cows (Bustad *et al.*, 1965). The "old" radiation units of microcuries for quantifying the amount of radioactive material are used in the scale on the left and the "new" International System units of becquerels are used in the scale on the right. Rather than contaminate a large quantity of feed, the cows were given two feed pellets each day, the feed pellets all contained  $5\mu\text{Ci/l}$  (185 Bq) of  $I^{131}$  on the first day of the study. The  $I^{131}$  decayed with a physical half-life of 8.06 days so the cows ingested less  $I^{131}$  each day (the straight line in the figure). The cows' thyroids and milk were monitored for  $I^{131}$  content. Aliquots of the  $I^{131}$  contaminated milk were ingested by five volunteers and their thyroids were monitored for  $I^{131}$ . As an aside, the calculated radiation dose to the thyroids of the volunteers was less than what



Reprinted with modification from Bustad, McClellan and Garner, "The Significance of Radionuclide Contamination in Ruminants", pp 131-146, in *Physiology of Digestion in the Ruminants*, 1965, Butterworth, Inc.

**FIGURE 2.5** Inter-relationships for radioiodine ( $I^{131}$ ): intake by cows, cow's thyroid and milk and human thyroid (adapted from Bustad *et al.*, 1965).

would have been received from an  $I^{131}$  thyroid uptake study conducted for diagnostic purposes. Let me hasten to note that the control limits for radionuclides in milk intended for human consumption would be sufficiently stringent that the radiation dose to the cows would be much lower than required to produce toxicity in the cows. These results have been used to estimate allowable intakes from  $I^{131}$  contaminated milk and to develop guidance for monitoring pasture and hay to control milk contamination. Data such as presented in Figure 2.5 were useful input to the control of radiation exposure of Japanese populations from ingestion of milk following the Fukushima, Japan, accident in 2011, as they were earlier for European populations following the Chernobyl accident.

## Toxicokinetics

The simple schematic rendering shown in Figure 2.1 can be used to illustrate several important concepts. First, it is important to recognize that contrary to common usage, exposure and dose are not the same. The exposure environment is characterized by the concentration of the toxicant in the media, be it water, air or feed, the quantities taken in and the time course of the intake. Dose is the concentration, over time, of the toxicant in the various tissues of the subject, whether it be a cow, a human or a laboratory rat.

For example, in Figure 2.5 the exposure of the cows would be the quantity of  $I^{131}$  ingested. An example of dose would be the measured quantity of  $I^{131}$  in the thyroid of the cows and human volunteers. A more precise estimate of dose would be the calculated radiation dose in rem (old radiation unit) or sievert (new radiation unit).

The information presented in Figure 2.5 may be viewed as being a limited toxicokinetic study. The key data acquired were the intake of  $I^{131}$  and the changing concentration of  $I^{131}$  in the cow's thyroid and milk. The information on the relationship between  $I^{131}$  intake and milk content are sufficient to provide input for estimating limits of daily intake of  $I^{131}$  in forage by the cows in order to limit the  $I^{131}$  content of milk to some prescribed limit such as an Intervention Level as will be discussed later. Because the physical half-life of  $I^{131}$  is quite short, 8.06 days, and the  $I^{131}$  is concentrated in the thyroid and milk, concern does not develop for the  $I^{131}$  content of the rest of the carcass. If the radionuclide or specific chemical were found in edible portions of the carcass, then information on the radionuclide, chemical or metabolite of the chemical in the edible tissues such as muscle would be of interest. For example, radio-caesium (caesium behaves like potassium) concentrates in muscle and is also present in milk. Radio-strontium (strontium behaves like calcium) concentrates in the skeleton and milk.

The characterization of the kinetics linking exposure with dose is referred to as toxicokinetics (for a toxic agent) or pharmacokinetics (for a pharmaceutical). In actual practice, the term pharmacokinetics is frequently used when it would be more appropriate to use the term toxicokinetics. Several chapters in this book deal specifically with kinetics of toxicants and pharmaceuticals.

Toxicokinetics (see Figure 2.1) are used to describe the movement and disposition of the toxicant in the organism. This includes consideration of the route of entry; ingestion, inhalation, dermal or purposeful administration by injection. A complete description of the toxicokinetics of a toxicant will take into account: (1) the intensity and duration of the exposure, (2) the rate and amount of absorption of the toxicant from the site of entry, (3) the distribution of the toxicant within the body, (4) potential biotransformation to less, equal or more toxic form, and (5) the rate of excretion by route (urine, feces or exhalation). All of these aspects of toxicokinetics may be influenced by species differences in physiological and biochemical characteristics. Modern approaches to modeling toxicokinetics attempt to take account of both species differences and similarities in influencing the fate in the body of toxicants. It is also important to recognize that the exposure or dose level may influence the kinetics of a toxicant and its metabolite(s). This is an especially important consideration in extrapolating from laboratory studies that may be conducted at high doses to lower more environmentally relevant exposures/doses.

## Toxicodynamics

The linkage between dose and adverse health outcome shown in Figure 2.1 involves multiple mechanisms as various toxicants may potentially impact all the cells and organ systems of the body. Increasingly, scientists have attempted to model these relationships which, in parallel to the nomenclature for the kinetic phase, are called toxicodynamic or pharmacodynamic models. It is obvious that multiple pathways may be involved in a toxicant producing disease and that knowledge of the individual steps will increase as knowledge of basic biological mechanisms increases. For example, the explosion of knowledge of basic biology at the level of the genome (genomics), proteins (proteomics) and metabolism (metabolomics) has provided a basis for exploring the mechanistic basis of toxicant-induced disease with a degree of refinement that could not even be envisioned even a short time ago.

A later chapter reviews the basic mechanisms of toxicity. In addition, many of the chapters on organ toxicity and specific toxicants contain detailed information on mechanisms of toxicity. As the reader reviews this material, and especially the detailed discussion of



biochemical mechanisms of action, it will be important to place those in the context of processes at the cellular and tissue level; i.e., inflammation, cell death, cell proliferation, hypertrophy, hyperplasia, metaplasia and neoplasia. A strength of the veterinary medical curriculum, as with the human medical curriculum, is the emphasis given to understanding both normal body processes and disease processes extending from the molecular level to cells to tissues to organs and, ultimately, to the integrated mammalian organism. A special opportunity exists for medically trained personnel, both veterinarians and physicians, to put the expanding knowledge of molecular and cellular processes into the context of overt disease. After years of emphasis on a reductionist approach to basic biomedical science, it has become recognized that this approach needs to be complemented by an integrative approach. This has recently been termed systems biology. In my view, this is not really a new concept. It is more a rediscovery and refinement of the concepts of integrated biology and pathobiology used in veterinary medicine for decades.

There has been great enthusiasm for the use of mechanistic information in safety/risk evaluations as will be discussed later. Recognition of the difficulty of characterizing all of the individual mechanistic steps has given rise to a new term – mode of action. The mode of action has been defined as the dominant step(s) involved in producing a given toxic endpoint. An example is the role of cell killing as the mode of action for large intakes of chloroform (Butterworth *et al.*, 1995) or formaldehyde (Conolly *et al.*, 2004), over extended periods of time causing tumors in rodents. The exposure–response relationship for cell killing may likely have a threshold which must be considered in extrapolating the findings from high exposure-level studies in rodents to humans exposed to low concentrations of these chemicals. Readers interested in a contemporary view of the use of mode of action information to improve regulatory decision making will be interested in a summary paper by Carmichael *et al.* (2011).

It is my contention that understanding the basic concepts conveyed in Figures 2.1, 2.2 and 2.4 can be very useful in investigating a range of situations where the objective is to establish or refute a causal association between a given source and toxic agent and an increased incidence of an adverse health outcome. The term increased incidence is used advisably, recognizing in most situations involving domestic animals, either as commercial herds or as companion animals, that the situation is one of presence or absence of a given disease and the “ruling out” of other differential diagnoses. However, in situations involving human populations the issue frequently encountered is whether a given toxicant exposure has caused an increase in a disease, recognizing that most diseases may have multiple etiologies, for example

hypertension and diabetes. This is especially the case in evaluating diseases that typically occur late in life, such as cancer and chronic diseases, and with exposure to toxicants that may occur at low levels over long periods of time. In some cases, such as lung cancer and cardiorespiratory disease in humans, a risk factor such as cigarette smoking is so substantial it is a challenge to determine if low-level exposure to other toxicants such as air pollutants is having chronic effects at low exposure concentrations.

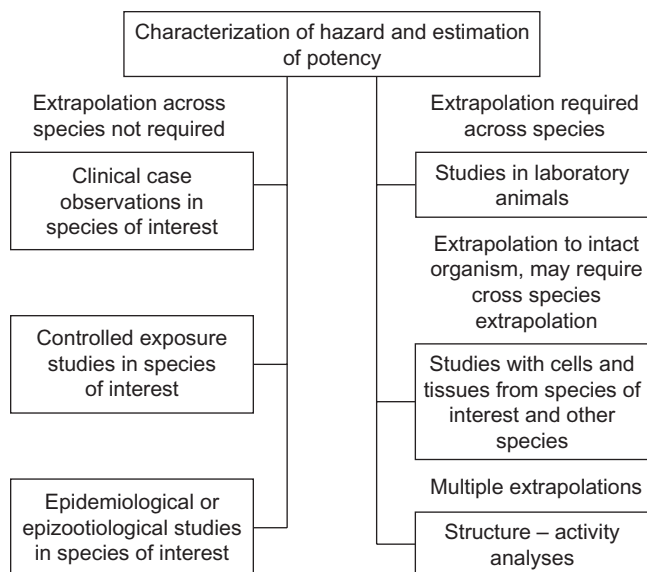
### Veterinary toxicology is multi-faceted

It will be apparent to the reader of this book that veterinary toxicology is multi-faceted. Thus, there are many ways to organize and synthesize the knowledge base that we call veterinary toxicology. One dimension is the various classes of toxicants. Another dimension of the field relates to the media that contains the toxicant; air, water, soil and feed. Another dimension considers the various routes of exposure of toxicants; inhalation, ingestion, dermal or purposeful injection. It is also convenient to consider the various organ systems and processes that may be affected by toxicants. This is the basis for organization of a major section in this book. It is also important to consider the individual toxicants or classes of toxicants. This approach is used in organizing another major section of this book. Finally, veterinary toxicologists recognize the necessity of considering the various species of concern. Increasing veterinary medical practitioners have become more specialized with many focusing their clinical skills on a single species. This book does not include a section addressing the toxicology of individual species. To have done so would have substantially increased the size of this text. However, chapter authors have endeavored to discuss species variations in responses to toxic agents. It is noteworthy that several textbooks, those of Petersen and Talcott (2006) and Poppenga and Gwaltney-Brant (2011), focus on small animals. Some of the major comprehensive veterinary medicine texts, such as the *Current Veterinary Therapy* series, which focus on other species include chapters on toxicology related to that species.

## SOURCES OF INFORMATION

### Case observations in the species of interest

There are multiple sources of scientific information for characterizing the relationship between exposure to a toxicant and toxicant-induced response. Figure 2.6 is a schematic rendering of the multiple sources of information that may be used to understand the toxicity of a given agent.



**FIGURE 2.6** Sources of information for evaluating potential toxicants.

As discussed earlier, the origins of veterinary toxicology and toxicology in general, are both rooted in observations. An adverse health effect, either a pattern of morbidity or death in an individual or population, is observed and the disease linked to exposure to a toxicant. Typically, the time interval between exposure and the adverse health outcome was brief which aided in deducing an association. Because the causal association was identified in the species of interest, whether it be a person, a horse or a cow, it was not necessary to extrapolate between species. Nor was it necessary to explore in-depth the mechanistic basis for the causal association to either diagnose a particular case or prevent future cases. Action to prevent exposures and, thus, prevent disease could be based on empirical observations.

As you read many of the chapters in this book, you will note that details of the mechanism by which a particular toxicant causes disease have been elucidated to a variable extent. When the toxicant is exclusively of concern in veterinary medicine and has no implications for human health, there has been limited impetus for developing a mechanistic understanding of how a toxicant causes disease. Concern for human health has been a major driver of the biomedical research agenda. An obvious exception is when the toxicoses observed in veterinary medicine have large economic impact or toxicants can reach people via animal products.

There are many circumstances where observational knowledge is not adequate and it is necessary to conduct experiments to characterize the toxicology of an agent. It is obvious that if concern for the potential toxic response is in a non-human species, controlled experiments

can be conducted using the species of interest. This is obviously the case for domestic livestock as well as companion animals.

A much more common situation is when concern focuses on potential toxicity of a newly developed agent for use in people or animals. For example, it is necessary to establish the safety of a potential new pharmaceutical or consumer product before it is introduced into commerce. In these instances experimental animals are used as a “first approximation” of the safety of the new compounds to humans. In the case of products intended for use in animals, studies on both efficacy and safety can be conducted in the species of interest. This remains an imperative step in the safety evaluation of new products. There are also circumstances in which it is desirable to extend limited observations from opportunistic studies on people or animals that have been exposed. When a new product is developed and marketed, either a pharmaceutical or a consumer product, various post-marketing surveillance systems should be put in place to attempt to detect any unexpected adverse outcomes.

### Epidemiological/epizootiological studies

If a particular chemical has been used for an extended period of time and human exposure has occurred either in the workplace or from the environment, it may be feasible to conduct epidemiological studies. Epidemiology is the study of how disease is distributed in the population and the factors that influence or determine this distribution. The design of a particular epidemiological study will be guided by the hypothesis being tested and the nature of the population(s) available for study. As an aside, the term epidemiology (epi for across, dem for people and ology for scientific study) is applicable to people while the more appropriate related term for studies on animals would be epizootiology (epi for across, zoo for animal and ology for scientific study). The details of conducting epidemiological or epizootiological studies are beyond the scope of this chapter. A relevant reference for basic concepts in epidemiology is the text by [Gordis \(2008\)](#).

A classic paper by [Austin Bradford Hill \(1965\)](#), a British medical statistician, provides guidance on the interpretation of epidemiological evidence for a given agent or factor causing a health effect ([Table 2.1](#)). Although developed for interpreting epidemiological data, i.e., data acquired on human population, the guidance is also highly relevant to interpreting data acquired on animal populations. The framework needs to be used intelligently rather than slavishly – it is a guide and not a checklist.

Retrospective epidemiological studies may be feasible for previously introduced agents for which prior exposure has occurred or prospectively for a newly introduced agent. If the agent is new it is obvious that it is not feasible

**TABLE 2.1** Bradford Hill (1965) guidance for interpreting epidemiological evidence for a given agent or factor causing a specific disease

*Strength:* If there is a large effect size (a strong effect), this makes a true causal relationship more likely. There is no fundamental reason why a strong association should be more likely to be causal than a weak association. However, Bradford Hill was thinking of possible confounding factors and argued that if a strong association is not causal, an association with some other factor that varies closely with the factor originally suggested must exist. If the association is strong, this co-variable will be easier to recognize than if the association is weak.

*Consistency:* Is there evidence for the same findings from more than one study, preferably from different settings?

*Specificity:* Is the effect specific to the exposure? In reality this rarely occurs, and some regard this as the “icing on the cake” when considering causal evidence. However, the specificity of a particular mechanism, if seen across a range of health endpoints, would meet the requirements of this component.

*Temporality:* This is the only absolute. Exposure must precede outcome.

*Biological gradient:* Is there a dose–response relationship? In addition (although explicit in Bradford Hill’s original paper), is there a threshold of exposure below which an effect is not seen?

*Plausibility:* Does this exposure–effect relationship make biological sense? Is there mechanistic evidence to support the likelihood that this might occur given current knowledge? Bradford Hill pointed out that this feature should not be demanded: what is implausible today may be entirely plausible tomorrow.

*Coherence:* Does the proposed causal association cohere with other findings? For instance, if we were concerned that a specific exposure might be related to a health effect that was based on an inflammatory response, has a similar effect been seen with other inflammatory conditions, or is an apparent effect on mortality accompanied by effects on morbidity?

*Experiment:* Is there evidence from experiments (involving either animals or humans, whether individually or as populations) that removal of the exposure reduces the effect?

*Analogy:* Are there analogous situations that would tend to support the likelihood of a causal relationship? For instance, when considering a possible teratogen, consideration of the effects of thalidomide on the developing fetus would come to mind.

to conduct epidemiological studies to retrospectively evaluate the potential safety/hazard of the agent. If the ultimate interest is in the effects on people, it may be feasible to conduct controlled exposure studies with human volunteers. It is advisable for the planning of such studies to be based on a solid database on the potential toxicity of the agent acquired from studies in laboratory animals. The design and conduct of such human studies must be guided first and foremost by ethical considerations (NRC, 2004). If a non-human species is the target species of concern, then it is obvious that the most relevant information is that acquired from studies conducted in that species.

## Experimentation

An additional option for acquiring information is to conduct toxicological studies in typical laboratory animal species. Such studies are the cornerstone of research conducted to evaluate the safety/risk of newly synthesized agents whether they be a potential new pharmaceutical, pesticide or herbicide, a significant consumer product or a new chemical or intermediate to be used in commerce. It is well recognized, certainly by veterinarians, that no single laboratory animal species is a miniature version of the human species, i.e., 15 cm in height, weighing 180 grams and sharing all of the common biological traits and diseases of humans. Fortunately, humans and laboratory animals do share many common biological traits and diseases. Knowledge of the extent to which there are similarities and differences between humans and a given

laboratory animal species can be used to guide the selection of a species to serve as a surrogate for humans in developing data for safety/risk evaluations for humans. It is encouraging that some veterinary medical schools are recognizing the importance of extending the range of species studied in the core curriculum from the usual companion animal and domestic livestock species to include the common laboratory animal species.

At this juncture, it is appropriate to note the importance of animal welfare issues. The Animal Welfare Act (AWA), initially enacted in 1966 and amended in 1970, 1976, 1985, 1990 and 2002, is the principal federal statute in the U.S. governing the sale, handling, transport and use of animals. The AWA applies to all species of warm-blooded vertebrate animals used for research, testing or teaching excluding animals used for agricultural research. The U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS) has responsibility for implementing the AWA. The 1985 Amendments to the AWA clarified the importance of humane care, minimization of pain and distress, consideration of alternatives, the role of institutional animal care and use committees, the psychological well-being of primates and exercise for dogs. The primary reference on animal care and use is the *Guide for the Care and Use of Laboratory Animals* prepared under the auspices of the Institute of Laboratory Animal Resources of the National Academy of Sciences/National Research Council (ILAR, 2010). All toxicologists involved with laboratory investigations should be familiar with the contents of the Guide irrespective of the species they use for their research.

An additional matter the experimentalist should be aware of is the need for use of good laboratory practices (GLPs) in the conduct of research intended to be used for regulatory decisions. Both the FDA (FDA, 2001) and the EPA (TSCA, 1985; FIFRA, 1991) have requirements for the use of GLPs. The FDA GLP requirements do not extend to exploratory, mechanism of action or efficacy studies. The basic elements of GLPs are: (1) the appointment by the institution of a study director, (2) the use of an independent quality assurance unit, (3) the use of documented standard operating procedures, (4) a written protocol for each study, and (5) preparation of a final report containing a GLP compliance statement for each study. The use of GLPs is not required by FDA for studies with domestic livestock. However, investigators conducting studies using domestic livestock would be well advised to attempt to adhere to the basic principles that undergird GLPs to help ensure the quality and reproducibility of the data being generated.

Another option for acquiring useful toxicity data is to conduct investigations *in vitro* using tissues or cells from mammalian species, both humans and laboratory animals, and using bacteria and yeasts. An additional option is to conduct structure–activity analyses on the new agent using the large data bank of structure–activity information already available on other related chemicals.

All of the options outlined, to some extent, create extrapolation issues. Even if studies are conducted in the species of interest, it is typically necessary to extrapolate from the high levels of exposure or administered doses studied experimentally to lower exposures or doses anticipated to be representative of intended use. It may also be necessary to extrapolate from a relatively short period of study, say days or a few weeks, to the intended period of use, over months or years. If the studies are not conducted in the species of ultimate interest, there is need to extrapolate between species. It may also be necessary to extrapolate observations made in a population of healthy individuals to a population that includes individuals with pre-existing disease or altered susceptibility to development of disease. Some aspects of the extrapolation between species and across exposure/dose levels may be facilitated by physiologically based toxicokinetic and toxicodynamic modeling. However, toxicodynamic modeling is still in its relative infancy.

It is important to recognize that even with today's level of knowledge of these extrapolation issues, it is not possible to estimate, with absolute certainty, the precise numerical level of human exposure to a given agent that may be without any risk of potential harm or will produce a specific level of harm. This is generally recognized in contemporary safety/risk evaluation methodology such that conservative approaches are used in estimating safe levels of human intake of chemicals. By taking a conservative approach to setting standards or providing

guidance to limit exposures, there can be a high degree of confidence that an agent can be used safely if used as intended. Ultimately, all processes that develop guidance or standards to limit exposures and thus limit disease require judgments to be exercised. In short, science can inform the standard or guidance development process; however, it cannot prescribe specific standards.

## Schematic experimental designs

The experimental design for testing of any specific hypothesis must be matched to the hypothesis, the desired statistical power and the resources available. Inevitably, decisions on an experimental design involve making difficult choices among options because of resource constraints. In this section, two schematic experimental designs will be discussed to illustrate some of the key issues that must be addressed in planning toxicological studies. The discussion in both cases will assume that the species to be used in the study has already been selected.

## Acquiring toxicokinetic data

The first conceptual design, Figure 2.7, illustrates an approach to acquiring data for understanding the link between exposure and internal dose, the kind of data that can be used for toxicokinetic modeling. Recall the toxicokinetic linkage in Figure 2.1. The design shown is based on a single brief intake of the test agent. However, the design can be modified for studying chronic intake of an agent. A critical decision is the choice of the route of administration or intake of the test material. Obviously, such studies are most readily carried out with parental administration of the agent. This may be the most appropriate route for a pharmaceutical agent that is to be parentally administered. However, the resulting data may be of limited relevance to other routes of intake. For example, it may not appropriately mimic oral

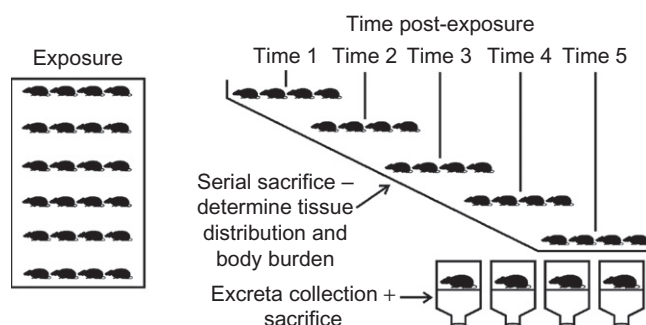


FIGURE 2.7 Schematic rendering of an experimental design for evaluating the kinetics of an administered toxicant.



intake since only a small fraction of some toxicants may be absorbed from the gastrointestinal tract. In short, the route of administration should be matched to the route of concern for real-world exposure to the agent.

With inhalation, the particle size distribution of the airborne toxicant will influence what portion of the inhaled material will be deposited and where it is deposited in the various regions of the respiratory tract. The pattern of retention and subsequent translocation of the deposited material will depend on the size, chemical composition and dissolution properties of the deposited material. Individuals interested in details on the conduct of inhalation toxicity studies will find the book edited by McClellan and Henderson (1995) of interest as well as a summary chapter by McClellan *et al.* (2006).

Another key decision is whether conduct of the toxicokinetic studies may be facilitated by using a test agent labeled with radioactive or stable element tracers. Analytical considerations for the initial toxicant as well as any metabolite are of major importance in the conduct of toxicokinetic studies.

The schematic design (Figure 2.7) shows a group of animals maintained for collection of excreta and, perhaps, even sampling of expired air. Data from these analyses can be used along with tissue analyses to obtain a mass balance between the quantity administered and the quantity recovered. The schematic design shows multiple times at which animals will be euthanized and tissues collected for analysis. This allows the development of a dynamic profile of how the body handles the administered material. For organic compounds, provision needs to be made for analyzing both the parent compound and potential metabolites.

The selection of the sacrifice times will be guided by the anticipated kinetic profile of the agent and its metabolites. It may be useful to obtain preliminary information on retention kinetics from pilot studies. Some organic compounds may be rapidly metabolized leading to the need to schedule all of the sacrifices over a few hours. On the other hand, certain inhaled relatively inherent materials may have long-term retention in the lungs extending over hundreds of days. It is important to recognize that the quantity of material administered may influence the kinetics of the material. Hence, it is desirable to use multiple administered exposure/dose levels as an experimental variable. Without question, the design of any particular toxicokinetic study requires the exercise of considerable professional judgment. Toxicological research is not a “cookie cutter” or “check the box” science.

### Acquiring exposure (dose)–response data

A schematic experimental design for a study to evaluate exposure (dose)–response relationships for toxicants

is shown in Figure 2.8. Recall the exposure–response linkage shown in Figure 2.1. The design shown is typical of that which might be used in the conduct of a 2-year bioassay, typically to evaluate carcinogenicity in rats and mice. The same design, and indeed the same experiment, can be used to evaluate other endpoints and to conduct shorter-term studies. The study should involve administration of the material by a route matched to likely exposure conditions to be encountered with the agent. Administration of an agent by gavage may be acceptable as a surrogate for ingestion, especially when it is desirable to administer specific quantities of material. However, I am not enthusiastic about the repeated use of gavage as a substitute for ingestion of an agent in feed. The use of intratracheal instillation as a surrogate for conducting inhalation exposures to an agent remains controversial. It is my professional opinion that intratracheal administration is a non-physiological mode for delivery of materials to the respiratory tract. It may result in exaggerated quantities of material being deposited in some regions of the respiratory tract while other regions are spared any exposure. This unusual pattern of distribution of the agent is very likely to influence the toxic responses of the airways and alveoli. Thus, I am hesitant to even recommend intratracheal instillation for mechanistic studies; the mechanistic information acquired may be irrelevant to the inhalation exposure situations that are of concern for people or other species.

It is critical that exposure–response studies utilize multiple exposure levels, perhaps three or four exposure levels. The choice of the specific exposure levels is one of the most important decisions to be made in planning such studies. One consideration relates to the potential level(s) of exposure to be encountered with intended use. Higher additional levels can be selected above this base level. Selection of exposure/dose levels can also be informed by the results of the kinetic studies. For example, it would not be desirable to use only exposure levels above a level at which metabolic processes are saturated. Another

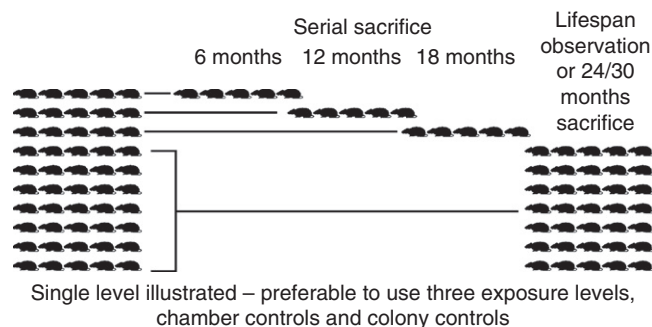


FIGURE 2.8 Schematic rendering of an experimental design for evaluating exposure (dose)–response relationships for a toxicant.

consideration emphasized by the EPA and NTP, especially when cancer is an endpoint, is to select a maximum tolerated dose (MTD) level as the highest exposure/dose level and establish lower levels by some fraction of the MTD level, perhaps 1/2 and 1/4 or 1/3 and 1/9. The use of an MTD has been justified on the grounds that it is necessary to maximize exposure to potentially observe carcinogenic responses recognizing the blunt experimental approach (NRC, 1993). A useful review on the history of the use of animal bioassays to predict carcinogenicity has been authored by *Beyer et al.* (2011).

The extent to which animal bioassays are a blunt approach to detecting the carcinogenic potential of agents is illustrated in *Figure 2.9*. It can be noted that for a species and strains of animals with a background incidence of 1%, a study of 50 animals will require a 20% response to detect a statistically significant effect. As an aside, a population of non-smoking people will experience about a 1% lifetime incidence of lung cancer. A population of a two pack a day cigarette smokers will experience about a 20% lifetime incidence of lung cancer compared to the 1% expected in non-smokers. Consideration of statistical information such as these emphasizes the importance of using care in interpreting the results of cancer bioassays using the typical 100 animals per exposure level. The interpretation of the relevance of the results of animal studies for estimating human hazards will be greatly enhanced by knowledge of the mechanisms involved in the toxicant causing disease in the animals.

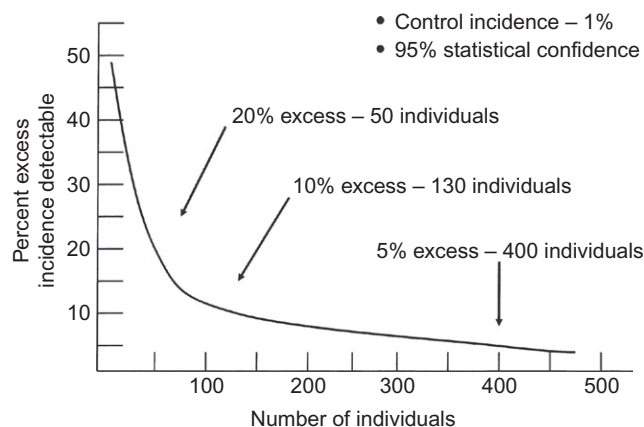
A key feature of the exposure–response experimental design illustrated in *Figure 2.8* is the use of multiple sacrifice times for all exposure levels. In some cases it may be possible to evaluate the functional status of organs at these times, i.e., pulmonary function. In animals with inhalation exposure, when a respiratory tract response is of concern, it may be feasible to collect bronchoalveolar

lavage fluid samples for analysis of biochemical and cellular parameters. Most importantly, tissue samples can be collected for histopathological evaluation. The information obtained from the serially sacrificed animals, combined with that obtained from the terminal sacrifice animals, can provide valuable insight into the progression of disease processes over the course of the study. Without question, insight into the pathogenesis of toxicant-induced disease processes will be much more complete when serial sacrifices are conducted than that obtained only from an evaluation of the terminal sacrifice animals. Another option in the design of exposure–response studies is to include a group of animals at each level that are removed from further exposure at one or more times post-inhalation of exposure for maintenance without further exposure. These animals may be euthanized at later times and evaluated for evidence of recovery or reversibility of earlier toxicant-induced changes.

The basic guidance for using multiple exposure (dose) levels and making experimental observations at multiple times is as applicable to the conduct of studies examining hypotheses on the mechanisms of action of toxicants as it is to studies developing information for regulatory decisions. I remain disappointed at the number of published papers on mechanisms of action of specific toxicants that fail to use multiple exposure (dose) levels and multiple observation times. It is only when exposure (dose) level and duration of exposure are included as experimental variables that a true understanding of the mechanisms of toxicity for an agent can be elucidated. Mechanisms are frequently exposure (dose) level and exposure duration dependent.

As the science of toxicology has advanced, increasing attention has been given to developing specialized approaches for evaluating toxicity induced in different organ systems. The various guidelines developed by the USEPA, FDA and NTP are useful references for these specialized approaches. For example, the EPA has published guidelines for evaluating carcinogenicity (EPA, 1996a), gene mutation (EPA, 1996b), reproductive toxicity (EPA, 1996c), developmental toxicity (EPA, 1991) and neurotoxicity (EPA, 1995). The EPA is continually reviewing and updating its guidelines for toxicity testing. Forty-nine harmonized health effects test guidelines used in the testing of pesticides and toxic substances have been developed and can be found on the EPA Office of Prevention, Pesticides and Toxic Substances website (EPA/OPPTS, 2006).

The FDA has provided specific guidance for evaluating the safety of compounds used in food-producing animals (FDA, 1994) and principles for evaluating the safety of food ingredients (FDA, 2011). The EPA has provided guidelines for evaluating the safety of products intended for use with cats and dogs (EPA, 1998) and domestic livestock (EPA, 1996d).



**FIGURE 2.9** Relationships between number of subjects required to detect excess risk and the level of detectable excess risk.

A special issue of concern with pharmaceutical products is the presence of trace contaminants and whether these trace contaminants should be tested individually. One approach to addressing this issue is the use of a “threshold of toxicological concern” concept championed by Kroes *et al.* (2005). Kroes, now deceased, was a Dutch veterinarian who developed an excellent reputation in safety and risk assessment. The concept of “safety qualification thresholds” (Ball *et al.*, 2007, 2011) developed out of the “threshold of toxicological concern” concept. It is a promising approach to minimizing the use of laboratory animals in exhaustive testing while still assuring the safety of pharmaceuticals. The European Medicines Evaluation Agency (EMA, 2004) has provided detailed guidelines for addressing genotoxic impurities.

The various guidelines are useful for planning safety evaluation studies. However, the guidelines should not be used as a substitute for the use of professional judgment in planning, conducting and interpreting toxicological investigations. As noted earlier, toxicology is not a “cookie cutter” or “check the box” science.

## TOXICOLOGIC DESCRIPTORS

### Toxicology rooted in observations

The results of toxicological investigations, either from clinical case observations or planned experimentation, involve describing the exposure, the dose, the response and inter-relationships between these parameters. Exquisite knowledge of exposure or dose or response is not sufficient. Ultimately, it is necessary to understand their inter-relationships. With clinical case observations, the initial emphasis is on the clinical findings – what are the response and the need, on the basis of a differential diagnosis, to establish that a toxicant is or is not involved. The evidence for a specific toxicant may be based initially on clinical findings complemented by gross necropsy findings potentially buttressed by histopathological findings. The differential diagnosis of a toxicosis may be strengthened by evidence of a marker of dose, i.e., urine, blood or tissue levels of suspected toxicant. The diagnosis may be further strengthened with evidence of exposure, i.e., the presence of the toxicant in the feed or identification of a poisonous plant. At each step, the qualitative evidence of a toxicosis and a specific toxicant is enhanced as qualitative findings are supplemented by quantitative findings. The analysis is not completed there, though. Other reasonable differential causes of the same or similar clinical signs must also be “ruled out” if the animals or humans are in a real world or field setting.

### Quantifying exposure

Quantitation is paramount in evaluating exposure. In the experimental setting, quantitation is considered beginning with the design of the study and continuing through all aspects of the experimentation. To the extent feasible, exposure to the toxicant should be rigorously characterized. This starts with physical and chemical characterization of the test material, be it an alleged pure compound or a mixture, including identification of any contaminants. The exposure circumstances need to be rigorously characterized. This, of course, is easiest to do when the test material is administered by injection. Even with injection, care must be taken to ascertain that the desired quantity of toxicant was actually injected. The quantity administered is typically related to the body weight of the subjects.

With administration by routes other than injection, the situation becomes more complicated. This may involve providing the experimental subjects’ feed to which the toxicant has been added. If this approach is used, samples of the contaminated feed should be collected periodically for analysis of the test agent. In some cases, the concentration of the test agent in the feed will be used as a measure of the exposure. To accurately quantify exposure, it will be necessary to know the concentration of the test agent in the feed and also determine the quantity of the contaminated feed containing the test agent that has been ingested. For dermal administration, it is necessary to know the concentration of the test agent in the liquid media applied to the skin and the quantity of the media applied to the skin.

The situation is much more complex for a test agent in the air, whether it is a diluted gas or suspended particulate material. In both cases, it will be necessary to sample and measure the concentration of the test agent in the air at a location as close to the breathing zone of the experimental subjects as possible. For both particulate material and reactive gases, there may be substantial loss of the test agent in the delivery system between the generator used to create the test atmosphere and the breathing zone of the subject(s). Care needs to be taken to minimize such losses. For a toxic agent in a particulate matter form, it is essential to know not only the concentration of the test agent, but the size distribution of the particulate matter since the aerodynamic particle size distribution will influence the fraction of the inhaled material that will be deposited and where it deposits in the respiratory tract. In some experiments, it may be possible to use a plethysmograph to measure respiration of individual subjects during inhalation exposure. This is most readily accomplished when the exposure period is relatively brief as in a study of the toxicokinetics of the agent. The total quantity of test agent inhaled can be estimated from knowledge of the air volume inspired and the concentration of

the test agent in the air. In many studies the air concentration of the test agent may be used as a surrogate measure of exposure. As indicated earlier, exposure and dose are not synonymous. However, in many studies it may be necessary to use the concentration of the test agent in the feed, water or air as a surrogate measure of dose.

## Describing absorption, distribution, metabolism and excretion

A number of different parameters may be evaluated in assessing the kinetics of a test agent (recall Figure 2.7). Some of the common parameters and terms used are shown in Table 2.2 adapted from Spoo (2004). The four key events involved are absorption, distribution, metabolism and excretion. It is important to recognize that species differences may exist for each of these events. Absorption is the amount of the material that enters the body. As already discussed, the concept is simple. However, in reality it becomes complex as one moves from parental administration to oral intake, to dermal uptake, or inhalation exposure. Distribution of the material will be influenced by the route of entry and the physicochemical properties of the test agent. Metabolism for compounds varies dependent on the physicochemical properties of the material. In some cases, the material

may be very inert and simply be transferred mechanically within the body with some portion excreted over time. In other cases, especially with organic compounds, the metabolism may be quite complex and result in metabolites that are either more toxic, less toxic or have toxicity similar to the parent compound.

Excretion or elimination of the material and its metabolites, if metabolized, may occur via the kidney (urine), gastrointestinal tract (feces), or the lungs (exhalation of volatile compounds). In addition, the agent or metabolites may appear in tears, sweat or exfoliated skin. Some species, such as the rat, may engage in coprophagy, ingestion of feces, such that the test material in the feces is ingested and some portion passes through the body multiple times. Animals may be euthanized at various times during the course of the study and samples of various tissues collected and analyzed for the test agent or metabolites. With small experimental subjects, it may be possible to analyze all the tissues and obtain an estimate of the total body burden of the test agent and metabolites.

In some short-term studies it may be possible to collect and analyze excreta and expired air, if the compound is metabolized to a form that will be present in expired air. This information, along with the results of tissue analyses, can provide an estimate of the total quantity in the body, excreta and expired air for comparison with an estimate of the quantity administered. This kind of mass

TABLE 2.2 Common terms used to describe the ADME characteristics of chemicals (adapted from Spoo, 2004)

Term	Abbreviation	Definition
Concentration	$C_p$	Concentration of a chemical in plasma (p) at a specific time (t)
Time	$t$	Chronological measurement of a biological function
Half-life	$t_{1/2}$	Time required for exactly 50% of a drug to undergo some defined function (i.e. absorbed, distributed, metabolized or excreted)
Volume of distribution	$V_d$	Unitless proportionality constant that relates plasma concentration of a chemical to the total amount of that chemical in the body at any time after some pseudo equilibrium has been attained
Volume of distribution (steady state)	$V_{d(ss)}$	Same as $V$ , except measured when the chemical has reached a steady state in the body
Area under the curve	AUC	Total area under the plasma chemical concentration curve from $t = 0$ to $t = \infty$ after the animal receives one dose of the chemical
Body clearance of a chemical	$Cl_B$	The sum of all types of clearance from the body
Renal clearance of a chemical	$Cl_R$	Volume of chemical that is completely cleared by the kidneys per unit of time (ml/min/kg)
Non-renal clearance of a chemical	$Cl_{NR}$	Volume of chemical that is completely cleared by organs other than the kidneys per unit of time (ml/min/kg)
Dose	D	The amount of chemical that is administered to an animal; can be further defined as the total dose, that total dose the animal was exposed to, or the absorbed (effective) dose, that being the fraction of the total dose that was actually absorbed by the animal
Bioavailability	F	Also known as systemic availability of a chemical. The quantity of percentage portion of the total chemical that was absorbed and available to be processed (CME) by the animal, in the case of intravenous administration, $F = 100\%$

ADME: absorption, distribution, metabolism and excretion; CME: chemical metabolism and excretion.



balance approach is obviously most feasible when radioactive or stable isotope tracers are used. One should not be surprised to find the estimated quantity recovered varying from 75 to 125%; there will be a high degree of experimental variability when multiple samples are being collected and analyzed. Obviously, one should view with suspicion data tables showing recovery of exactly 100% of the administered dose. Such values are typically the result of an overzealous investigator normalizing the data to 100% recovery. For chronic exposure studies, it may be possible to use kinetic modeling to estimate the quantity of the test agent or metabolites present in the experimental subjects at each exposure concentration at various times after initiation of exposure.

### Toxicant-induced responses

The types of studies typically used by toxicologists to investigate exposure–response relationships can be placed in four categories related to the duration of the studies; acute, sub-acute, sub-chronic and chronic (recall Figure 2.8). Acute studies are usually of a day or less and may involve intraperitoneal, intravenous or subcutaneous injection, gavage, dermal application or inhalation. Injections may be given once or several times in the 24-hour period. Acute inhalation exposures are typically 4 to 6 hours in duration. In all cases, the observations are made over a 24-hour period. Sub-acute studies typically involve repeated exposures made on a daily, or 5 days per week, basis for 2 to 4 weeks with observations over the same period of time. Sub-chronic studies are usually conducted over a period of 1 to 3 months. In the case of inhalation exposures, these are typically conducted for 4 to 6 hours per day, 5 days per week. Chronic studies are usually conducted for more than 3 months and, most typically, for 2 years. I personally view the use of the terms acute, sub-acute, sub-chronic and chronic as jargon and prefer to communicate the duration of studies in a specific manner, i.e., number of days or months, or as short or long term. I prefer to use the terms acute, sub-acute, or chronic as descriptors of a medical condition.

The kinds of toxicant-induced responses that may be encountered are broad, essentially mirroring the range of disease processes that may occur in humans and other animal species. In any well-conducted toxicity study, the investigator should use as broad an array of observational techniques as are reasonably available to characterize the pattern of morbidity and mortality that may develop. Inevitably, cost constraints will influence the choice of endpoints evaluated. It will be useful to prioritize the potential endpoints as to their likely value in terms of the information gained. It is crucial that detailed necropsies be conducted on subjects euthanized at prescribed times and at termination of the study. Tissues

should be collected from any gross lesions and tissues identified in the protocol as likely target tissues and processed for histopathological evaluation. It is now routine to establish a defined set of criteria for evaluating the various tissues and characterizing lesions. This approach allows the quantitative evaluation of any pathological findings on a group basis rather than restricting the evaluation to qualitative descriptions of responses in individual subjects.

Toxicity studies to evaluate exposure (dose)–response relationships may extend from minutes to hours when biochemical and physiological responses are being evaluated, to hours to days when acute morbidity and mortality are being assessed, to weeks to months and finally to a significant portion of the lifespan of the species, for example 2 years for mice and rats when chronic effects, including cancer induction. With increased attention given to animal welfare considerations, emphasis is being given to using as few animals as possible to define the acute morbidity and mortality of test materials. Rather than use a traditional approach to attempt to precisely define a lethal dose 50% ( $LD_{50}$ ), it has become customary to use approaches with many fewer animals to define an approximate  $LD_{50}$ . In some cases, it may be desirable to determine the concentration of a test agent in water or air that produces 50% lethality over a defined period of time, a lethal concentration,  $LC_{50}$ . This approach remains in common use when studying aquatic organisms.

In modern toxicology, increasing attention is given to conducting studies with exposures that are defined by the anticipated conditions of use of the test material. This may involve initially conducting a study of 2 weeks' duration, perhaps with up to five exposure levels anchored by a level related to anticipated use. The results of this study are then used to select exposure levels, perhaps three or four, and to sharpen the focus of a 90-day study. The results of the 90-day study, in turn, are used to select the exposure levels and sharpen the focus of a study of 2 years' duration. Although it has become customary to conduct chronic exposure or 2-year studies with three exposure levels, it should be recognized that use of a control group and three exposure levels spanning a range of concentrations differing by a factor of 2, i.e., 1, 1/2 and 1/4 or a factor of 3, i.e., 1, 1/3, and 1/9, does not provide a robust data set for characterizing the shape of the exposure (dose)–response relationship. On the other hand, the use of exposure levels differing by a factor of 10, i.e., 1, 1/10 and 1/100 may provide an excessively broad range of exposure levels for identifying a lowest observed adverse effect level or no observed adverse effect level as will be discussed later.

In chronic studies, major attention is directed to evaluating any toxicant-induced changes in animals at the several exposure levels compared to controls over

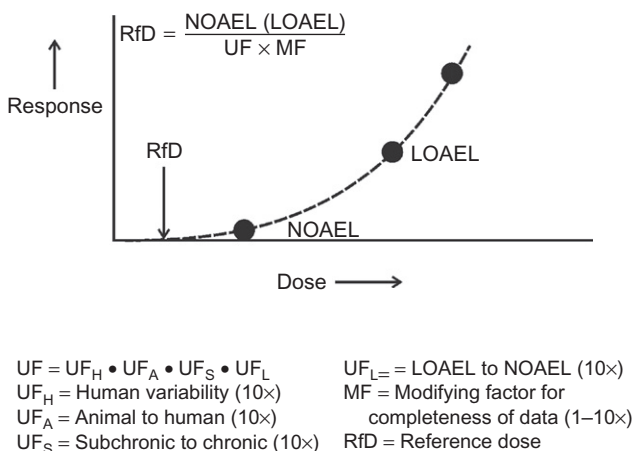


a 2-year period or until a defined mortality level is reached, such as 20% surviving. Any changes in various indices of morbidity or pathological alterations will be evaluated compared to controls as well as tested for trends across the exposure levels. In many cases, the primary endpoint of concern will be cancer which should include evaluation of all stages of tumor development up to sarcomas and carcinomas. It has become customary to use life table statistical methods such as that of [Kaplan and Meier \(1958\)](#) to evaluate the incidence of key changes. This approach allows for the use of data not only from the survivors at the end of the study, but also animals that have died or been euthanized at interim times. This situation is analogous to that encountered in human epidemiological studies when subjects may be lost to follow-up.

It has become customary when the results of chronic studies will be used for regulatory purposes to convene a pathology peer review panel of expert veterinary pathologists, typically Diplomates of the American College of Veterinary Pathology (ACVP), to evaluate histological specimens from representative cases and the diagnoses of the original pathologist to verify that the diagnoses are appropriate and consistent with the scientific norm. As an aside, I encourage veterinary toxicologists to personally review the pathology findings in studies with the study pathologist so as to be familiar with the nature of the pathology findings. However, I discourage veterinary toxicologists from taking on a dual role of toxicologist and pathologist for a study. Indeed, this approach would be unacceptable for a study to be submitted for regulatory purposes unless the toxicologist was also an ACVP Diplomate.

### Describing exposure–response relationships for non-cancer endpoints

It is now appropriate to consider how the data generated from toxicological investigations can be used. Let us first examine a threshold exposure–response relationship as shown in [Figure 2.3](#) and shown now in an expanded form in [Figure 2.10](#). The first step is to examine the data set from critical exposure–response studies to identify key parameters to be used to describe the results. Key determinations are the no observed effect level (NOEL), the highest exposure level for which no effects are observed and the no observed adverse effect level (NOAEL), the highest exposure level that produces no adverse effects. Obviously, characterization of an effect as adverse or not adverse is a matter of professional judgment. For example, in a cholinesterase inhibitor study, is a reduction in blood cholinesterase in the absence of salivation or other clinical signs an adverse effect or merely an effect?



**FIGURE 2.10** Schematic rendering of a threshold–exposure response relationship.

In the absence of the identification of a NOAEL, there is a need to identify the lowest observed adverse effect level (LOAEL), the highest exposure level at which an adverse effect is observed. The specific NOAEL and LOAEL that can be identified are a function of the exposure levels originally selected for studies. To state the obvious, observations can only be made at the exposure levels studied. For example, if the exposure levels studied did not extend to a sufficiently low level, the lowest level might produce an effect thereby precluding observation of a NOAEL. Alternatively, the study might be designed with three exposure levels separated by a factor of 10 with the lowest exposure level identified as a NOAEL and the next higher exposure level identified as producing some modest adverse effects and, thus, identified as the LOAEL. In retrospect, in such a study it is not known whether the “true” LOAEL might have been a factor of three or five above the NOAEL since these levels were not investigated.

Another consideration is the nature of the effects identified at the NOAEL; was there evidence of enzyme induction or hyperplasia, hypertrophy or atrophy with no evidence of a change in organ weight? Likewise, at the LOAEL were hyperplasia, hypertrophy or atrophy present resulting in modest or substantial changes in organ and body weight? Were histological changes observed that were reversible? Were the changes sufficiently profound that the level would be identified as a functional effect level (FEL)? These questions serve to emphasize the extent to which professional judgment is involved in interpreting the results of all toxicological investigations.

For non-cancer effects a reference dose (RfD) for oral intake or an inhalation reference concentration (RfC) for airborne materials is calculated using the NOAEL or LOAEL as a starting point ([Jarabek et al., 1990](#); [Jarabek, 1994](#)). An RfD or RfC may be defined as an estimate (with

uncertainty spanning perhaps an order two magnitude) of a continuous oral or inhalation exposure to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious non-cancer effects during a lifetime. The RfD and RfC values are developed from the experimentally determined NOAEL or LOAEL values as shown in Figure 2.10 (Jarabek, 1994) and normalized to continuous exposure. For a more complete description of the process, the reader is referred to a recent book chapter by McClellan *et al.* (2006). The EPA maintains an Integrated Risk Information System that includes comprehensive summaries of the toxicological information available on specific chemicals including RfD and RfC values and estimates of cancer-causing potency. These profiles are available online (EPA/IRIS, 2011).

A somewhat similar approach for non-cancer effects has been used by the American Conference of Governmental Industrial Hygienists (ACGIH) to develop threshold limit values (TLV) (ACGIH, 2011). A TLV is defined as airborne concentrations of substances that represent conditions under which it is believed that nearly all workers may be repeatedly exposed day after day without adverse health effects. Since the ACGIH TLVs apply to healthy workers they may not always incorporate a safety factor (SF) or uncertainty factor (UF) of 10 for human variability. The exposure duration for TLVs is based on a 40-hour/work week and, thus, the results of animal studies will be normalized to 40 hours/week.

The Agency for Toxic Substances and Disease Registry (ATSDR) develops minimal risk levels (MRLs) using a similar methodology. An MRL is an estimate of the daily human exposure to hazardous substance that is likely to be without appreciable risk of adverse non-cancer effects over a specified duration of exposure. For example, MRLs are derived for acute (1 to 14 days), intermediate (14 to 365 days) and chronic (365 days and longer) exposure durations. The MRLs are intended to serve as a screening tool to help public health professionals decide to look more closely at particular exposure situations. The ATSDR has prepared toxicological profiles on many chemicals including their MRLs. More than 200 profiles are available online (ATSDR, 2006).

The National Institute of Occupational Safety and Health (NIOSH) develops recommended exposure levels (RELs). RELs are set at levels such that virtually all persons in the working population (with the possible exception of hypersensitive individuals) would experience no adverse effects. The Occupational Safety and Health Administration (OSHA) sets permissible exposure levels (PELs) based on consideration of the NIOSH RELs. However, the OSHA values are legally enforceable limits unlike the NIOSH RELs which are guidance.

The International Programme on Chemical Safety (IPCS) prepares authoritative reviews on the environmental health impact of various chemicals. The reports

are available online (IPCS, 2011). The exposure limiting values developed by the IPCS are guidance values and not legally enforceable limits. The United States makes extensive use of legal enforceable exposure limits. Many other countries emphasize the use of guidance values. This distinction is important when comparing standards versus guidance originating from different countries.

In considering all of the foregoing guidance or regulatory levels, it is important to recognize that they are set to control exposures for workers or the general public. In each case, they are set to be health protective and, thus, are set at levels below where human effects have been observed or are expected to occur. These values should not be interpreted as being equivalent to levels producing adverse effects in humans.

### Cancer as an endpoint

For cancer as an endpoint, animal exposure-response studies may provide two kinds of input. First, the results may be used in carcinogen classification processes such as those of the International Agency for Research on Cancer, the EPA or NTP. As discussed earlier, these are hazard-based classification schemes – is a given agent capable of causing human cancer without consideration of the potency of the agent? These schemes have been described elsewhere (McClellan *et al.*, 2006; McClellan, 1999, 2010).

If a positive cancer outcome is observed in animal studies, the quantitative exposure-cancer response data may be used in a second way – to develop a risk coefficient, lifetime cancer risk per unit of exposure, for the potency of the agent for causing human cancer. Such extrapolations typically involve linear statistical extrapolations from high levels of exposure used in the animal studies to potential human exposure levels several orders of magnitude lower (recall Figure 2.3). In addition, they may purposefully be calculated based on the upper 95% confidence limit on some level of risk, for example, with a probability of a one in one million occurrence. In my opinion, these extrapolated values are highly uncertain. It is quite possible that for some agents classified as possibly or probably carcinogenic to humans in the absence of a positive association with cancer from epidemiological studies and, thus, based on high exposure-level animal study results, there is no added cancer risk at very low levels of exposure (Gold *et al.*, 2003). The EPA (2005a) has issued guidance for alternative approaches to estimate cancer risks when information is available on the mode of action of the agent, for example if the cancer arises as a result of the toxicity and secondary cell proliferation rather than a direct effect of the chemical or metabolite on DNA. For example, chloroform has been shown to cause cancer by this mode of action (Butterworth *et al.*, 1995). The EPA (2005b) has also provided guidance for considering the impact of susceptibility of early life exposures for causing cancer.

Information on the cancer-causing potential of various chemicals is included in the material summarized in the USEPA's Integrated Risk Information System ([EPA/IRIS, 2011](#)). The IARC monographs on the evaluation of carcinogenic risks to humans are all available online ([IARC, 2011](#)). The monographs cover the carcinogen classification reviews of over 800 compounds. The NTP publishes, on a biannual basis, a Report on Carcinogens. The 11th Report contained 246 entries, 58 of which were listed as "human carcinogens" with the remaining 188 being listed as "reasonably anticipated to be human carcinogens" ([NTP, 2005](#)). The potency of the various agents for causing cancer is quite varied. When examining this literature, many in the public, including some scientists, are surprised to learn how few agents have been conclusively identified as "human carcinogens." The facts stand in sharp contrast to the view conveyed in the popular media and some scientific publications that people live in a "world of carcinogens."

### New potential endpoints

In recent years, the expansion of knowledge at the molecular and cellular level has provided the opportunity for considering the addition of a myriad of new endpoints to toxicological evaluations. This includes an array of new molecular biomarkers which have received substantial attention. Although biomarkers are frequently discussed as new approaches, it is well known to the veterinary clinician and toxicologist and to physicians that biomarkers have been used in both human and veterinary medicine for centuries.

In some cases, measurement of the biomarkers present in body fluids, urine or exhaled breath serve as an indicator of exposure or, even, dose of a toxicant. Recall the report of the individual arrested for "driving while intoxicated" based on a breathalyzer test for exhaled alcohol which has been converted to a blood alcohol level. In other cases, the biomarker is an indicator of a disease process. Recall individuals being evaluated for prostate cancer based on an elevated level of prostate-specific antigen in serum samples.

New biomarkers of exposure will continue to be proposed. For each potential biomarker of exposure, it will be necessary to conduct experiments to validate the utility of the biomarker. A special challenge relates to recognizing the dynamics of the toxicokinetics of various toxicants and establishment of quantitative relationships between exposure and dose at any particular time over the course of the intoxication.

The potential list of biomarkers for toxic responses is seemingly endless. In all fields of medicine, from different kinds of cancer to various functional diseases of every organ system, new molecular markers are being

identified on a regular basis. The challenge for toxicologists is to consider from among this array of opportunities which biomarkers are sufficiently well validated with regard to their linkage to diseases and sufficiently reasonable in cost to warrant their use in exposure-response studies. This includes consideration of the new and highly sophisticated genomic tools. There is a special challenge in designing validation studies to make certain that the experimental design is directed toward identifying specific disease-related endpoints or toxicant-related effects rather than merely being another, albeit more sophisticated, marker of non-specific toxic effects. A serious issue in many previous validation studies has been the use of a single high exposure level and a few short-term observation times. Such studies are unable to evaluate exposure-related changes in biomarkers and may not be able to identify toxicant-specific changes.

Two recent reports prepared by Committees of the National Research Council provide insight into how some individuals envision the future of toxicity testing. The first [NRC Report \(2007a\)](#) addresses the application of toxicogenomic technologies in predictive toxicology and risk assessment. The second [NRC Report \(2007b\)](#) provides a strategy and vision for toxicity testing in the 21st century. I encourage veterinary toxicologists, whether they are in training or experienced, to carefully read these reports. I view both of the reports as aspirational. In my opinion, both committees would have benefited from participation by veterinarians who understand the complexity of disease processes and toxicoses to counterbalance the views of molecular and cell biologists who focus on the micro-level and systems biology and may overstate the role of mathematical models. The concept of systems biology has come to the forefront in recent years. In my view, the concept of a systems approach to normal biology and disease processes has been a fundamental concept undergirding both veterinary medicine and human biology for more than a century.

## CONCLUSIONS AND SUMMARY

Veterinary toxicology is a multi-faceted hybrid that draws on and contributes to the veterinary medical profession, the scientific field of toxicology and, broadly, to medical science. Some have characterized toxicology as a distinct scientific discipline. I view toxicology as an applied area of science addressing important societal issues by drawing on multiple scientific disciplines and professions. Veterinary toxicology, as a sub-specialty in veterinary medicine, had a very applied origin – the diagnosis and treatment of toxicoses in domestic animals and companion animals. That important role continues

today. However, the field has broadened to include concern for contaminants in human food products originating from animals and for contributing to the conduct and interpretation of safety/risk evaluations for pharmaceuticals, food additives, consumer products and specific chemicals. Veterinary toxicologists who understand both normal and disease processes extending from the molecular level to the integrated mammalian organism and, indeed, populations, have an array of opportunities for making significant contributions to society. The prospects for the future of veterinary toxicology and the opportunities for veterinary toxicologists have never been brighter.

## DEDICATION

This chapter is dedicated to the memory of Charles C. Capen (1936–2008), an internationally recognized comparative pathologist and endocrine toxicologist. A complete obituary was published in *Toxicologic Pathology* (Anonymous, 2008). Chuck and I were classmates in the College of Veterinary Medicine at Washington State University receiving our Doctor of Veterinary Medicine degrees in 1960. Chuck immediately entered the residency and graduate program in Veterinary Pathology at the Ohio State University (OSU). He received his Ph.D. from OSU in 1965 and soon passed the certifying examination of the American College of Veterinary Pathologists (ACVP) and became an ACVP Diplomate. He progressed rapidly through the academic ranks becoming a full professor and, ultimately, a distinguished university professor. He provided outstanding leadership as chairperson from 1981 to 2002 of the OSU Department of Veterinary Biosciences. He was an outstanding teacher and researcher. One of his major contributions was in the interpretation of endocrine system effects observed in laboratory animal species as to their significance as predictors of human disease for various chemicals, including candidate pharmaceuticals. His special expertise resulted in his being asked to serve on many advisory committees and as a consultant to many companies around the globe. He was an elected member of the Institute of Medicine of the National Academy of Science and a recipient of many other honors.

## ACKNOWLEDGMENTS

Many of the concepts presented in the chapter are based on my experience working with talented scientists at three institutions; the Hanford Biology Laboratory at Richland, WA, the Lovelace Inhalation Research Institute (now a part of the Lovelace Respiratory Research Institute) in Albuquerque, NM, and the Chemical

Industry Institute of Toxicology (now the Hamner Institutes Health Sciences) in Research Triangle Park, NC. Moreover, my insights into the basic concepts of toxicology have been sharpened by my experience serving as an advisor on toxicology and human health risk issues to public agencies and private clients and the opportunities I have had for working with many outstanding toxicologists and other scientists.

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# Toxicokinetics

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## INTRODUCTION

The epithelial barriers that form the body's interface with the environment, and the cellular barriers that separate internal organs and tissues, have to be permeable to a wide variety of chemicals to allow nutrient absorption and waste product elimination. The permeability of these physiological barriers, however, also allows toxicants to penetrate. Exposure to toxicants through ingestion, inhalation, and dermal contact may therefore result in harmful absorption. The degree of adverse effects following exposure to a toxicant is dependent on the rate and extent of absorption into the body, distribution to susceptible organs and tissues, and specific interactions with biological targets. Living organisms are adapted to toxicant exposure through the development of transport and elimination mechanisms that prevent the build-up of harmful chemical concentrations in organs and tissues.

Toxicokinetics refers to the movement and fate, also referred to as the disposition, of toxicants. The term is most commonly used when describing the time course of absorption, distribution, and elimination (including biotransformation and excretion) of toxicants in an organism. Toxicokinetics is closely related to pharmacokinetics, and may even be viewed as the same discipline, with the only difference being the class of compound (toxicant or pharmaceutical) that is involved. Compared to typical pharmaceutical exposure, exposure to toxicants is often uncontrolled and variable, and very large doses may be involved. Toxicants are also more likely to cause lesions and abnormal physiological function that may alter the toxicant disposition, compared to exposures to pharmaceutical drugs below toxic levels. When very large doses are involved, kinetic

processes that can become saturated are more likely to reach their maximum rates, resulting in altered disposition.

Predicting tissue concentrations over time is essential for the prediction of adverse effects ([Andersen \*et al.\*, 2006](#)). It is also needed to prevent unwanted xenobiotic residues in animal tissues from entering foods of animal origin. Kinetic parameters, such as duration of internal exposure and tissue concentrations, are therefore useful metrics for assessing risk. Mathematical models are used to characterize these parameters by describing changes in toxicant concentrations over time. Models are most often used as predictive tools following exposure to toxicants, but they can also be used as an aid to understanding the underlying physiological mechanisms of chemical absorption, transport and elimination. Various types of toxicokinetic models have been developed and the appropriateness of a specific model type depends on the available data and the model's purpose (Riviere, 2011). One of the key challenges to the successful use of models is identifying the appropriate conditions for its application. Two fundamentally different types of toxicokinetic models are recognized: traditional (also called "classic") toxicokinetic models and physiologically based toxicokinetic models. Traditional toxicokinetic models are mathematical descriptions of concentration/time profiles that are constructed without assuming that the compartments and functions used in the models are directly representative of physical structures or physiological processes. The selection of compartments and functions are therefore solely dependent on their ability to simulate experimentally observed concentration/time profiles. Physiologically based toxicokinetic models, on the other hand, are mathematical simulations of kinetic processes in organs and tissues. The mathematical constructs therefore represent actual organs, tissues and physiological processes.

## UNDERLYING PHYSIOLOGY

### Absorption

Most hazardous substances must gain access to the systemic circulation to exert their toxic effects through interaction with one or more internal organs. Notable exceptions are those compounds that cause a local reaction at the site of exposure. Absorption is the process whereby toxic substances gain entrance to the body from the external environment by crossing cellular barriers. The primary routes of exposure for toxic substances are oral, respiratory, and dermal.

The gastrointestinal, respiratory, and dermal systems are lined with epithelia that present significant barriers to the entry of foreign substances due to tight junctions between their cells, or continuous lipid layers in the case of skin. The membranes of cells that form viable epithelial barriers are traversed by transporter proteins that either actively exclude xenobiotics, or facilitate the movement of specific substrates across the barrier. The onset, duration and intensity of a substance's toxic effects are therefore dependent on the toxicant's ability to permeate lipid cell membranes directly, and its interactions with transporter proteins. Dermal penetration is unique in the sense that the outer epithelial cellular layers (corneocytes) are non-viable and do not contain transporter proteins. Absorption, in this case, is therefore dependent on the ability of toxicants to penetrate the intercellular lipid matrix found between corneocytes. There are several factors that may influence the permeability of epithelial barriers to specific xenobiotics, including the physicochemical properties of the substance, blood supply at the site of exposure, and the concentration of the substance at the site of contact. A specific compound may be categorized as relatively non-toxic by one route and highly toxic via another due to differences in absorption from these sites.

### Distribution

Distribution is the process whereby toxicants move throughout the body and reach their site of action. Once absorbed, a toxicant typically enters the interstitial fluid at the site of absorption and then passes into the tissue cells or enters the blood and/or lymph. Blood is moved rapidly through the body by the cardiovascular circulatory system and this process constitutes the major mechanism whereby absorbed chemicals are distributed to the various organs and tissues of the body.

To be distributed through the body by the cardiovascular system, a toxic chemical must first cross the capillary endothelium, and then diffuse through the interstitial fluid and penetrate the cells of the target

organ. The entrance of xenobiotics to some tissues is restricted by special barriers (e.g., blood–brain barrier, blood–testes barrier and blood–placenta barrier) that form continuous cellular layers with tight junctions that prevent movement of toxicants into tissues by passive diffusion through intercellular spaces. To gain entry into these protected tissues, toxicants must pass through lipid cell membranes, either by penetrating the lipid membranes directly, or by active or facilitated transport through transmembrane transporter proteins. Factors that determine a compound's rate and extent of distribution therefore include molecular size, lipophilicity, plasma protein binding and the ability to interact with transmembrane transporter proteins. Uneven distribution through the body may occur due to affinity for specific environments such as fat for highly lipophilic compounds or bone for compounds that bind to  $\text{Ca}^{2+}$ . This can lead to extremely low concentrations in the blood plasma and accumulation with prolonged storage of the compound at the depot sites.

### Biotransformation

Biotransformation is a key body defense mechanism whereby chemical reactions transform xenobiotic compounds in the body. The major transformation reactions for xenobiotics are divided into two phases. Phase I reactions (oxidation, reduction, hydrolysis and acetylation) modify the compound's structure by adding a functional group. This allows the substance to interact with Phase II enzymes, which conjugates it with a water soluble molecule such as sulfate, glucuronide, glutathione and amino acid. The water solubility of a compound is typically increased by this process, and it is an important step towards the excretion of lipid soluble toxicants. Water soluble compounds that are small enough to pass through the renal glomerulus can usually be excreted relatively rapidly through the urine without biotransformation. Biotransformed toxicants will often have reduced toxicity compared to the parent compounds. In some cases, however, biotransformation increases toxicity. Biotransformation enzymes have broad substrate specificity. They are, therefore, able to transform a wide range of substrates.

### Excretion

One of the primary mechanisms of protecting the body from the toxic effects of toxicants is the elimination of these compounds from the body. Compounds that are rapidly eliminated are less likely to accumulate in tissues and damage critical cells. Although the terms elimination and excretion are sometimes used synonymously, the former term encompasses all the processes that



decrease the amount of parent compound in the body, including biotransformation. Excretion is the term used specifically to refer to the processes by which toxic compounds leave the body through excretory organs. The main routes of elimination are urine, feces and exhaled air. Except for the lung, water-soluble substances are more readily excreted because they are not readily reabsorbed through cellular barriers in the excretory organs.

## TRADITIONAL (COMPARTMENTAL) TOXICOKINETIC MODELS

### Introduction

Traditional compartmental pharmacokinetic models describe the aggregate result of all the processes involved in determining the concentration/time curve of a compound in a reference compartment, which is most often the venous blood, and referred to as the central compartment. It uses single or multiple compartments and first-order rate equations, chosen to optimally describe experimental data, with no direct physiological relevance or fidelity to anatomical structure or physiology. The main use of these models is to predict plasma concentrations in exposure conditions that are similar to the conditions under which the data were produced from which the model was derived. Traditionally, exponential equations have been used to quantitatively describe the changes in concentrations of toxic substances in plasma and tissue over time Eqn (1) (Gibaldi and Perrier, 1982; Rivere, 2011).

The following is a typical bi-exponential equation used to describe time/concentration data of xenobiotics in plasma:

$$C(t) = Ae^{-\alpha \times t} + Be^{-\beta \times t} \quad (1)$$

where  $C(t)$  is the xenobiotic concentration at time  $t$ ,  $\alpha$  and  $\beta$  are the slopes of the two phases with different disappearance rates and  $A$  and  $B$  are their intercepts with the  $y$ -axis.

In these models, the body is viewed as comprising one or more “equilibrium compartments,” from which the toxic compound disappears at the same rate. These abstract compartments are not ascribed to specific organs or regions of the body, but are understood to encompass a collection of tissues with similar blood supply and affinity for the compound of interest. The number of exponential terms in these traditional compartmental kinetic models therefore reflects the number of kinetically homogeneous compartments, with an additional term added to account for absorption if exposure is extravascular. These exponential equations

then serve as the basis to calculate physiologically relevant pharmacokinetic parameters that reflect the various kinetic processes ( $V_d$  = volume of distribution;  $CL$  = clearance). The equations are also used to predict plasma concentrations for different exposure scenarios.

The most common models used to describe plasma time/concentration profiles of xenobiotics are the one- and two-compartment open models. The one-compartment model describes the profile of a compound that distributes instantaneously and evenly in the body and is eliminated at a rate that is proportional to the amount left in the body. On a semi-logarithmic scale, plasma concentrations of these compounds decline linearly over time (Figure 3.1). For other compounds, an additional compartment (exponential term) must be added, because the plasma concentrations decline in two phases, with the decline during the first phase typically being more rapid than during the second phase (Figure 3.2). Concentrations of these compounds in the second compartment rise, peak and subsequently decline over time as the substance is eliminated from the body.

This approach to toxicokinetic modeling assumes that absorption, distribution, metabolism and excretion occur

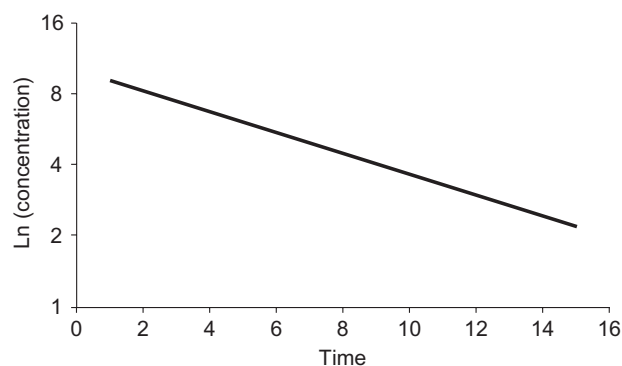


FIGURE 3.1 First order decline in plasma concentrations on a semi-logarithmic scale, according to a one-compartment model.

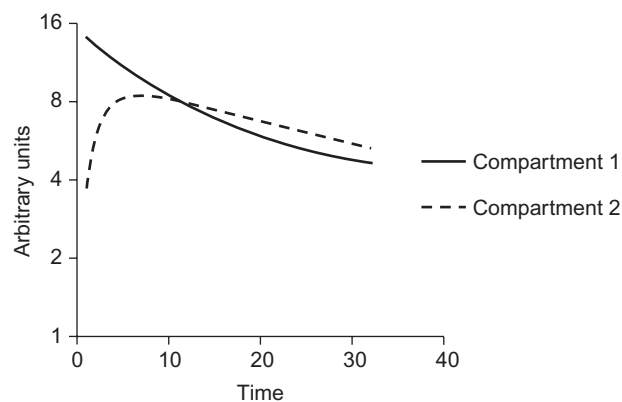


FIGURE 3.2 Plasma concentrations according to a two-compartmental model, demonstrating the separate contributions of two compartments to the rise and decline in blood concentrations.



at rates that are directly proportional to the concentration of the toxicant (i.e., that they are first-order kinetic rate processes). It is important to realize that this assumption does not always apply for toxic compounds, particularly at high concentrations when the transporters and the enzymes that facilitate these processes become saturated. This means that, in the absence of evidence showing that the system maintains linear, first-order kinetics over the range of concentrations that are of interest, the values of kinetic parameters should be interpreted with caution and cannot be used to extrapolate to higher or lower doses.

## Measures of absorption

Often, only a fraction of the total dose to which an animal or human is exposed gets absorbed systemically. This fraction is referred to as the bioavailability ( $F$ ) and is calculated by comparing the areas under the plasma time/concentration curves for the toxic compound administered intravenously versus the typical route of exposure. These data are not readily available for most toxic compounds, although the relative bioavailability from different routes (e.g., oral versus dermal) is often known. Other measures of the rate and extent of absorption include the absorption rate constant ( $k_a$ ), maximum measured concentration in the plasma ( $C_{\max}$ ) and time after exposure when this concentration is measured ( $T_{\max}$ ).

## Volume of distribution

The total volume of fluid in which a toxic substance must be dissolved to account for the measured plasma concentrations is known as the apparent volume of distribution ( $V_D$ ). If a compound is distributed only in the plasma fluid, the  $V_D$  is small and plasma concentrations are high. Conversely, if a compound is distributed to all sites in the body, or if it accumulates in a specific tissue such as fat or bone, the  $V_D$  becomes large and plasma concentrations are low. The value of this parameter is calculated from the multi-exponential equation that is fitted to the data using:

$$V_D = \frac{\text{Dose}}{\sum_{i=1}^n C_i} \quad (2)$$

which is the calculation of apparent volume of distribution from traditional pharmacokinetic parameters where  $C_i$  refers to the intercepts of the various phases of the curve with the  $y$ -axis.

## Clearance

Total body clearance ( $CL$ ) is the pharmacokinetic parameter that reflects the body's inherent ability to eliminate

a xenobiotic through organs of elimination. The value of this parameter represents the volume of blood cleared of the toxic substance per unit of time. If the total absorbed dose is known,  $CL$  can be calculated using Eqn (3). Many times the absorbed dose is not known, and the calculated value of this parameter reflects not only  $CL$  but also an unknown value for bioavailability ( $F$ ). The lower  $F$ , the higher the calculated value for a specific dose will be:

$$CL = \frac{\text{Dose}}{AUC_{0-\infty}} \quad (3)$$

which is the equation used to calculate total body clearance, where  $AUC_{0-\infty}$  is the area under the plasma time/concentration curve extrapolated to infinity.

## Half-life

A compound's half-life in plasma ( $T_{1/2}$ ) is a composite parameter that is dependent on both the body's inherent ability to eliminate the compound ( $CL$ ), as well as the extent to which the compound is distributed through the body ( $V_D$ ). This relationship is illustrated in the following equation:

$$T_{1/2} = \frac{0.693 \times V_D}{CL} \quad (4)$$

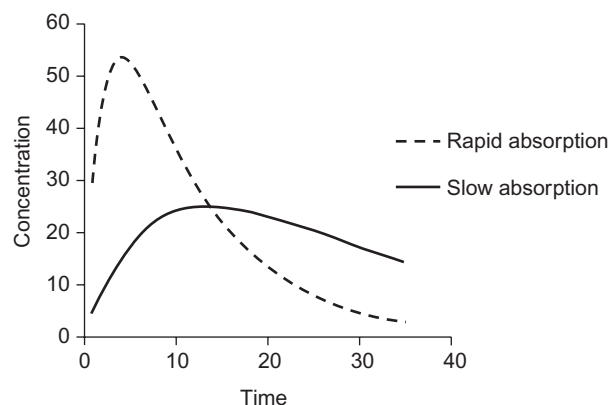
which represents widely distributed compounds due to their low concentrations in plasma (assuming first-order kinetics, where the rate of elimination is proportional to the amount of drug presented to the eliminating organ).

## Flip-flop kinetics

Flip-flop kinetics refers to a situation when the rate of absorption of a compound is significantly slower than its rate of elimination from the body. The compound's persistence in the body therefore becomes dependent on absorption rather than elimination processes (Figure 3.3). This sometimes occurs when the route of exposure is dermal.

## Residues

An additional concern with food-producing animals is the potential for adverse effects in the human consumer if edible tissues harvested from exposed animals contain harmful residues of toxic compounds. Tolerances (also known as maximum residue levels in countries other than the U.S.) are legal limits of allowable concentrations to prevent harm in consumers from toxic compounds in foods. The length of time following exposure required



**FIGURE 3.3** Plasma concentrations comparing a slow rate of absorption to a rapid rate of absorption, demonstrating “flip-flop” kinetics, where persistence of the compound is dependent on the rate of absorption, rather than the rate of elimination.

for concentrations in animal tissues to deplete to levels that are below these tolerances is referred to as the withdrawal time. The length of these withdrawal times are closely related to the compounds’ rates of elimination, and therefore their half-life, in the specific tissue of interest. It is important to note that the tissue depletion rate is unlikely to be the same as the rate of depletion from plasma, although the two are often related.

## PHYSIOLOGICALLY BASED TOXICOKINETICS

### Introduction

Physiologically based toxicokinetic (PBTK) models are mathematical simulations of physiological processes that determine the rate and extent of xenobiotic chemical absorption, distribution, metabolism and excretion. Such models can be used for predicting internal doses at target organs and tissues due to their conformity to actual organs, tissues and physiological processes. Internal dose predictions are useful for dose–response analyses and risk assessment involving specific mechanisms and sites of toxicity. Successes in the application of PBPK models to predict xenobiotic concentrations at target sites have led to its acceptance as a modeling technique in risk assessments. It is also used in mechanistic studies of the underlying processes that determine pharmacokinetic profiles and dose–response relationships.

In veterinary toxicology, PBPK methods can be used to improve the accuracy of predictions of toxicity across species by applying data obtained in one species to predictions in another species. It can be used to predict the effects of changes in physiological conditions, environmental conditions, activity levels and pathological

changes on xenobiotic concentrations in target tissues. This allows for more accurate assessment of risk in varied individual animals and populations. PBPK approaches can be used to study and understand the effects of mechanisms that determine the internal exposure of animals to potential toxins – such as dermal absorption and xenobiotic metabolism. PBPK models can also be used to address problems associated with the exposure of food producing animals to drugs and chemicals that may result in potentially harmful or undesirable residues in meat, milk and other foods of animal origin (Brocklebank *et al.*, 1997; Craigmill, 2003; Buur *et al.*, 2005).

PBPK models also make use of compartments, but in contrast to traditional compartmental models, the compartments are derived from mathematical descriptions of physiological body compartments or tissues. Links between the compartments simulate physiological processes of partitioning, transfer, metabolism and excretion. If the compartments and kinetic processes are accurately described, the concentration/time curves of chemicals in specific organs and tissues can be estimated. This offers an advantage over traditional compartmental models, because differences in physiology, anatomy, environment, metabolism and the effects of chemical-induced physiological changes and pathology can be simulated. Parameters can be scaled to reflect different dose ranges, species, breeds, genetic polymorphism and life-stages. The advantages of PBPK models are, however, difficult to achieve because the necessary anatomical and physiological parameter values are often not known, and/or the relevant pharmacokinetic processes are not well understood. The completeness of PBPK models depends entirely on the completeness of knowledge of the modeled system, and complete models are not attainable in most situations. PBPK models tend, therefore, to be simplified representations of reality based on assumptions regarding the most important processes and structures that determine the pharmacokinetic profile of the chemical in question.

Typical PBPK models simplify the body and represent it as a series of well-stirred compartments representing major organs and tissues of interest, a single dose and route of exposure, and the major route of excretion. The compartments are linked by blood flow, and the movement of chemicals between compartments is determined by tissue/blood partitioning and blood flow rates. However, when enough data are available, highly detailed, multiple compartment models can be constructed that include complete dosing regimens or exposure scenarios, detailed organ structures and physiological processes, as well as specific processes of metabolism, and simulations of metabolite pharmacokinetics. Models of varying complexity focusing on specific organs and routes of absorption/elimination, such as the skin and respiratory organs, have also been developed (Andersen *et al.*, 2002; Frederick *et al.*, 2002; Van der Merwe *et al.*, 2006).

## Model construction

The first step in the construction of a PBPK model is determining the purpose of the model and what internal tissue doses are needed to answer the specific scientific questions being asked. Once that is done, a schematic diagram is constructed that consists of each of the tissue compartments of interest, a plasma compartment, and a compartment or compartments that represent the rest of the physiological system. It is often necessary to include more than one compartment to represent the remaining portions of the body to reflect the differences in high and low blood flow tissues. The compartments can be subdivided into extracellular and intracellular spaces (Colburn, 1988). Tissue blocks and their sub-compartments can be combined to make the model as simple or as complex as needed. A schematic diagram for a generic whole body model is shown in Figure 3.4. Figure 3.5 depicts a generic tissue block with sub-compartments as well as possible binding sites and sources of elimination. In contrast to the full body model, Figure 3.6 presents a schematic diagram used in the prediction of sulfamethazine (SMZ) tissue residues in swine (Buur *et al.*, 2005). This model contains several simplifications, including a reduced number of tissue blocks, and the combination of tissue blocks into a single compartment. Because the model was designed to predict SMZ residues in edible swine tissues, only the edible tissues were specifically included in the model. The rest of the body was lumped into a single tissue block. Elimination is by renal filtration and is schematically represented using arrows that are not directed into the plasma compartment. The main metabolite is also modeled, and the two xenobiotics are linked together via the liver tissue block. This is an example of how model complexity can be optimized to achieve specific aims.

Following a schematic design of the model, the model is described using mass balance equations that represent the changes in tissue concentrations over time. The limiting factor in the exchange between compartments is an important consideration. In most models, the exchange is either limited by diffusion through a membrane barrier (diffusion-limited), or it is limited by the amount of blood flow (flow-limited). Both types of exchanges may be present in a single PBPK model. Diffusion-limited exchanges are often associated with large, polar molecules, and organs with small blood flow to mass ratios. Flow limited exchanges are most commonly associated with small, lipophilic compounds, and organs with relatively small volume and large blood flow to mass ratios.

The basic mass balance equation for a flow-limited compartment is:

$$V_t dC_t/dt = Q_t(C_a - C_v) \quad (5)$$

where  $Q_t$ ,  $V_t$  and  $C_t$  are the blood flow, anatomic volume, and concentration of the compound in the compartment,

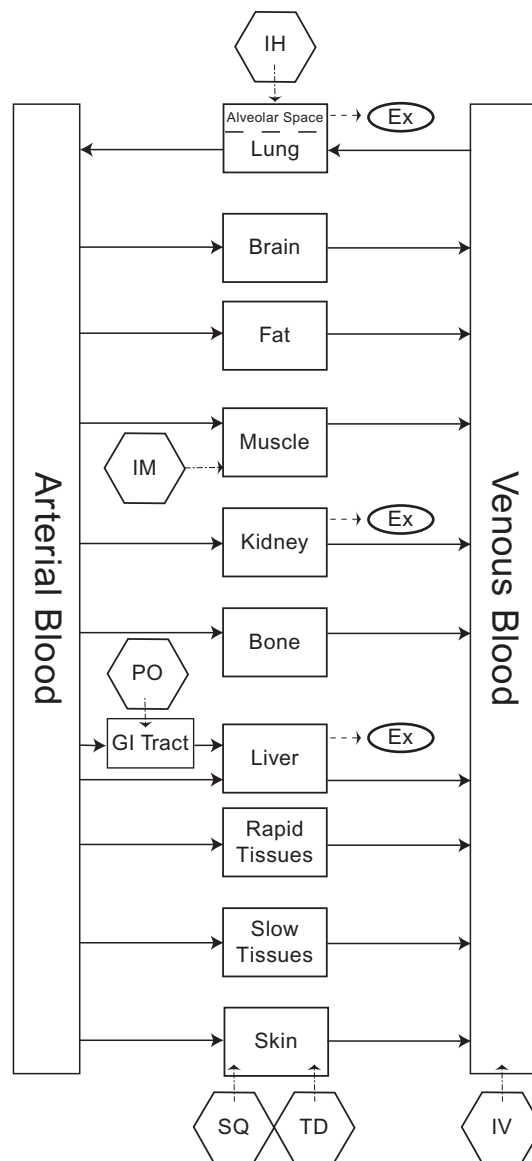


FIGURE 3.4 Schematic diagram of a generic whole body physiologically based pharmacokinetic model.

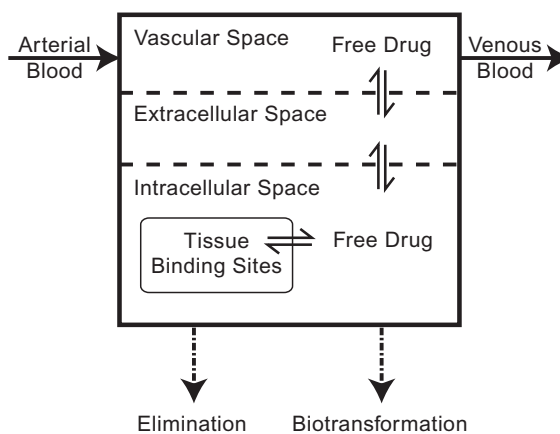


FIGURE 3.5 Schematic diagram of a generic tissue block physiologically based pharmacokinetic model, with sub-compartments as well as possible binding sites and sources of elimination.

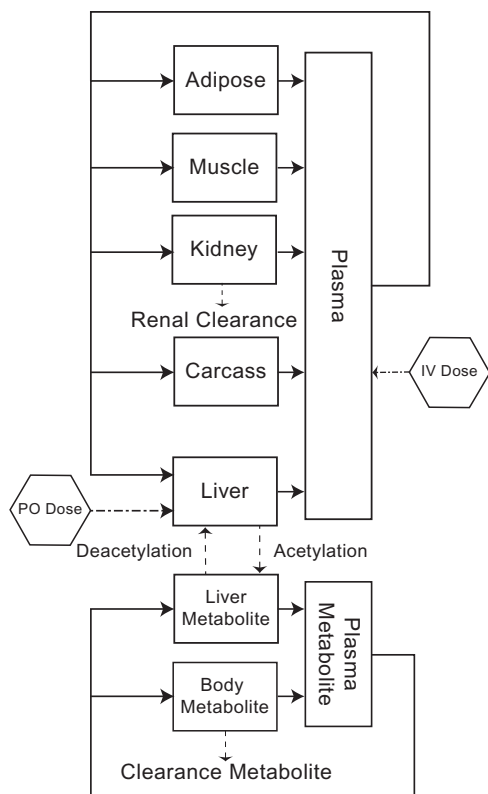


FIGURE 3.6 Schematic diagram of a physiologically based pharmacokinetic model, used in the prediction of sulfamethazine tissue residues in swine.

and  $C_a$  and  $C_v$  are the concentrations of the compound in the arterial and venous blood flowing into and out of the tissue. It is assumed in flow-limited models that the compound is in instantaneous equilibrium between the tissue and the blood, and that distribution in the compartment is homogeneous. This allows for the relationship between the venous blood concentration and the vascular space to be defined according to the partition coefficient between the tissue and blood:

$$C_v = C_t/P_t \quad (6)$$

where  $P_t$  is the tissue-to-blood partition coefficient. Therefore, the final mass balance equation is:

$$V_t dC_t/dt = Q_t(C_a - C_t/P_t) \quad (7)$$

This equation can be used in models where all tissue compartments are simplified into a single compartment. In contrast, membrane-limited exchanges do not assume that tissue concentrations are in equilibrium with venous blood concentrations. Instead, it uses sub-compartments representing extravascular and intracellular space. The assumption is that the vascular space is in equilibrium with the extracellular space. The mass balance equation

is then defined by rate of change in the extracellular space per unit of time, and can be written as:

$$\begin{aligned} V_e dC_e/dt &= Q_t(C_a - C_e) - K_t(C_e - C_i) \\ V_i dC_i/dt &= K_t(C_e - C_i/P_t) \end{aligned} \quad (8)$$

where  $V_e$  and  $C_e$  are the anatomic volume and concentration in the extracellular space,  $V_i$  and  $C_i$  are the volume of the intracellular space and the concentration in the intracellular space, and  $K_t$  is the membrane permeability coefficient for the membranes that separate the intracellular and extracellular spaces.

Further modifications are needed to describe metabolism or excretion. This can be done by adding a mass removal term,  $R_{ex}$ . The resulting equation for a flow-limited tissue block is:

$$V_t dC_t/dt = Q_t(C_a - C_t/P_t) - R_{ex} \quad (9)$$

The mathematical definition of  $R_{ex}$  can be as simple or as complex as needed to describe the important features of the process. Commonly used functions include simple first-order exchanges, and Michaelis-Menton equations that describe processes that can become saturated. For example, if  $R_{ex}$  is defined as the clearance of a specific organ the equation would be:

$$V_t dC_t/dt = Q_t(C_a - C_t/P_t) - C_t Cl_{organ} \quad (10)$$

where  $Cl_{organ}$  is the clearance of the eliminating organ.

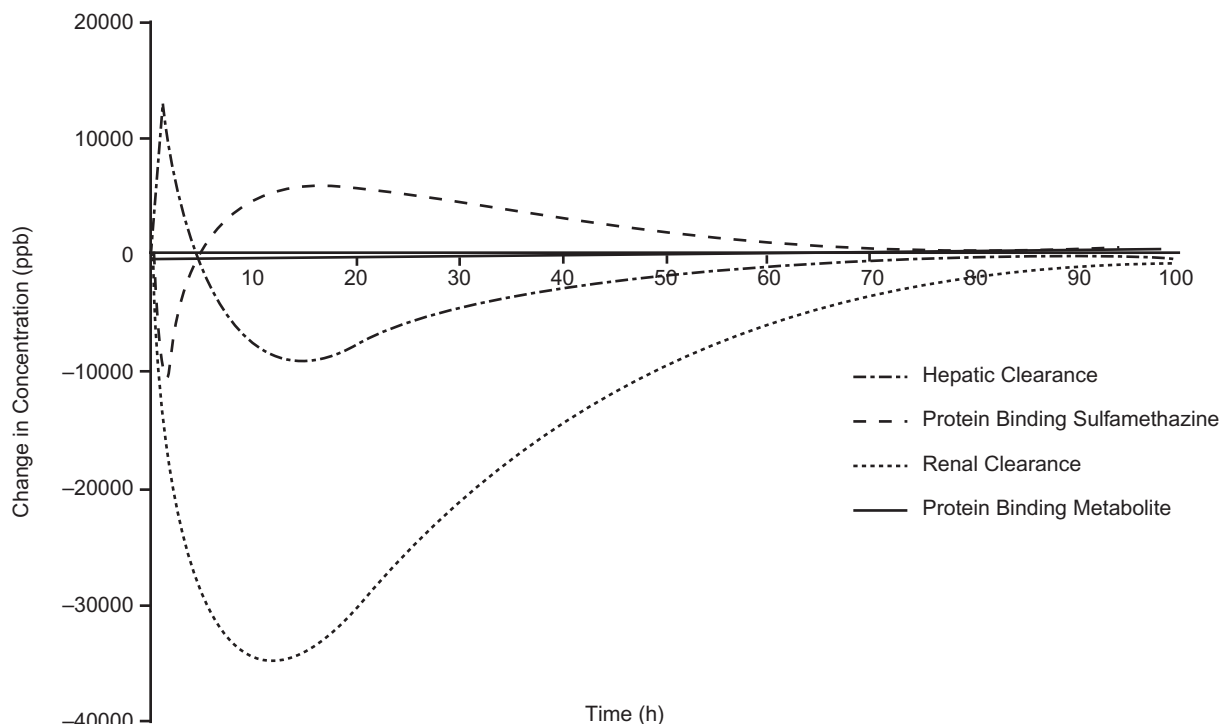
Tissue blocks can be further refined by the addition of modification terms to describe processes such as protein binding, tissue binding, active transport, biliary excretion, enterohepatic circulation, and metabolism. For example, if  $R_{ex}$  is governed by metabolism, and the appropriate parameter values of enzyme activity are known, it can be described using Michaelis-Menton equations such as:

$$R_{ex} = (V_m f_1)/(K_m + f_1) \quad (11)$$

where  $V_m$  is the maximum rate of metabolism,  $K_m$  is the concentration at which the rate of metabolism is 50% of maximum, and  $f_1$  is the free concentration in the metabolizing organ. The final step is to write the mass balance equation for the central (venous blood) compartment. The input into this compartment is the combined venous blood streams from the various tissue compartments. The rate of change in concentration in the central compartment is described by:

$$V_p dC_p/dt = \sum Q_t C_v - Q_p C_p \quad (12)$$

where  $V_p$ ,  $Q_p$ , and  $C_p$  are the anatomic volume, the total blood flow, and the concentration in the central



**FIGURE 3.7** A comparison of the relative contributions to plasma disposition from the parameters of renal clearance, hepatic clearance, and tissue partitioning coefficients.

compartment, and  $C_v$  represents the venous blood concentration from each tissue compartment. As with other compartments, this compartment can be modified to incorporate processes such as protein binding, or partitioning into blood cells.

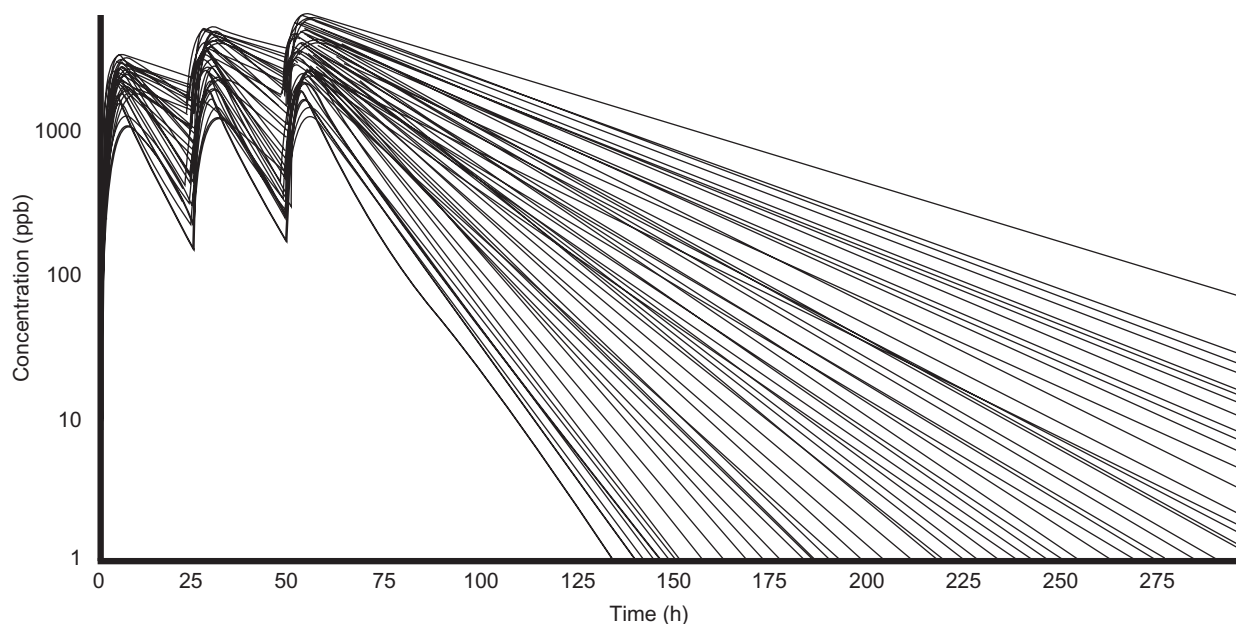
### Parameter estimation and identifiability

Accurate parameter values are essential for PBPK models to achieve their full predictive potential. The relevant parameters are often physiological (blood flow, organ volume, vascular space volume, etc.), and physicochemical (partitioning coefficients, membrane permeability coefficients, rate of absorption, etc.). Some parameter values can be estimated from *in vitro* experiments (protein binding rates, Michaelis-Menton constants, etc.). Many parameter values can be found in the published literature. Some parameters can be derived from *in vitro* and *in vivo* experimentation. However, there will usually be some parameters for which independent values cannot be obtained. These parameter values must be estimated using a curve-fitting process against known data points (Sheiner, 1985). Several curve-fitting software packages are available. Most use a function of a least likelihood ratio to estimate the parameters. It is important to emphasize that a weakness of PBPK models is their dependence upon a large number of parameters. The large numbers

of parameters can also make identifiability challenging. Identifiability refers to the ability to specifically determine a unique influence on model output for each parameter, based on an ideal data set. As the number of tissue compartments increases, the ability to uniquely identify all parameters is diminished without the inclusion of additional data points. Valid inferences cannot be drawn from a model if the model contains unidentifiable parameters. To reduce the likelihood of this type of error, multiple tissue compartments may be sampled. Thus, it is not only the number of data points representing the final model output that are used, but also the number of sampled compartments that is important for accurate parameter estimation (Audoly *et al.*, 2001). Identifiability problems can also be reduced by decreasing the number of parameters that need to be estimated. Sensitivity analysis can be used to decide which parameters can be abandoned without significantly altering model output. It compares the relative contributions of reasonable ranges of parameter values to an output of interest (Evans and Andersen, 2000). For example, in the SMZ model example, the relative contributions to plasma disposition from the parameters of renal clearance, hepatic clearance and tissue partitioning coefficients can be compared (Figure 3.7). The parameters of protein binding, hepatic clearance and renal clearance have the greatest effect at early time points.

The range of parameter values to use can be estimated using statistical distributions rather than fixed ranges.





**FIGURE 3.8** A Monte Carlo analysis using a physiologically based pharmacokinetic model, used in the prediction of sulfamethazine tissue residues in swine, to simulate multiple oral dosing.

As with single point estimations, the accuracy of the model is directly proportional to the accuracy of the distribution. In many cases, a reasonable mean and range of parameter value distribution can be inferred from published data. Distribution patterns can also be assumed to follow a model commonly found in natural systems (normal, log-normal, beta, etc.).

## Model validation

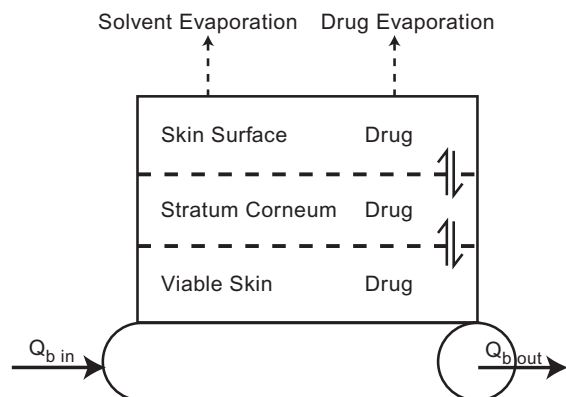
Model validation refers to the process of confirming that the model actually achieves its intended purpose. In most situations, this will involve confirmation that the model is predictive under the conditions of its intended use. This type of validation occurs by comparing model simulations to an independent experimental data set. Data used in the estimation of model parameter values cannot be included in the external data set. Simulated data derived from the model are compared to observed data points. The sets of data may be plotted side by side using simulation plots; or output values at specific times can be compared using correlation plots and residual plots. Results are then subjected to qualitative and quantitative analysis for goodness of fit. Unlike traditional compartmental pharmacokinetic modeling approaches, there is currently no standardized method to evaluate the goodness of fit for PBPK models. Often, a combination of visual examination of residual plots and simulation plots, along with the quantification of regression correlation values ( $R^2$  values), are used. In general, residual plots should have normal distributions around zero without

any time bias. Correlation plots should have regression lines with  $R^2$  values close to 1, and intercepts close to the starting value (in most cases, this is zero). Simulation plots are also used to detect time and concentration bias.

If a complex model was created by the incorporation of population distributions, then model validation typically becomes more qualitative in nature. In these cases, sampling methods such as Monte Carlo or bootstrapping can be used to generate specific values for the parameters in question. This parameter value assignment is repeated a large number of times, and the output becomes a set of simulations that can be plotted alongside each other. This gives a visual representation of what a population may look like (Sweeney *et al.*, 2001). Figure 3.8 shows a Monte Carlo analysis using the SMZ model to simulate multiple oral dosing (Buur *et al.*, 2006). The oral absorption rate, rate of gastric emptying, protein binding, and both renal and hepatic clearances were varied. Validation of these data is performed by plotting the multiple simulations alongside independent experimental data points. However, confidence in the distributions and in the model is determined by visual inspection, rather than correlation coefficients or residual plots. Generally, more data points covered within the spread of the output results in higher confidence in the predictive ability of the model.

## Applications

PBPK models are most often used in toxicology to predict the concentrations of toxic chemicals and their



**FIGURE 3.9** Schematic diagram of a physiologically based pharmacokinetic model used to describe the trans-dermal absorption of organophosphate insecticides in flow-through diffusion cells.

metabolites in target tissues. Target tissue concentrations predict toxic effects better than exposure concentrations or concentrations in a reference compartment such as venous blood. The adaptability of PBPK models makes them suitable for extrapolations across different exposure scenarios and routes, species, breed, age, physiological state, pathological changes and sex differences. PBPK models are also used in basic research to understand the effects and interactions between anatomical structure, tissue composition and physiological processes that influence the concentration/time course of xenobiotic chemicals. Toxicologists are often faced with situations where the adverse health risk from chemical exposure must be estimated without access to data obtained under the conditions of the exposure being analyzed. An advantage of well-designed, appropriately detailed and adequately understood PBPK models is that different exposure scenarios can be simulated using the same base model by varying the mathematical descriptions of the dosing regimen. These may include single, repeated or continuous oral ingestion, single or repeated intravenous boluses, constant intravenous infusion, single or repeated injections at various body sites, inhalation and topical exposures under various conditions. As long as the influences of dependent parameters on the reference concentration/time curve can be reliably identified, the model can be used to simulate the effects of variations in parameter values. It can also be used to do limited hypotheses testing related to key parameters. An example of this approach is the use of a PBPK model to describe the transdermal absorption of organophosphate insecticides in flow-through diffusion cells (Figure 3.9) (Van der Merwe *et al.*, 2006). Detailed independent parameter estimations, in a relatively simple physiological system, reduced the dependent parameters to three – each with an identifiable influence on the concentration/time

curve in the receptor chamber of the flow-through cell. Sensitivity analyses were used to identify important parameters and to generate hypotheses regarding the effects of changes in the skin related to those parameters. This approach can be used to discover important parameters *in silico*, which can be used to generate testable hypotheses. For example, the number of cell layers in the stratum corneum was identified as an important parameter and the effect of changing numbers of cell layers was simulated. The simulation could then be used as a hypothesis and tested using skin with varying layers of cells in the stratum corneum in flow-through cell experiments.

## CONCLUSIONS

PBPK modeling is an evolving frontier in toxicokinetic modeling. As our understanding of the systems and processes involved in toxicokinetics improve and expand, so does our ability to use newly gained knowledge in models. PBPK models allow for the adaptability needed to simulate varied physiological processes and system conditions. PBPK models are therefore likely to assume an ever more important role in our efforts to understand and predict the consequences of exposure to toxicants, and its application in veterinary toxicology can be expected to expand.

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# Factors affecting chemical toxicity

Carlo Nebbia

## INTRODUCTION

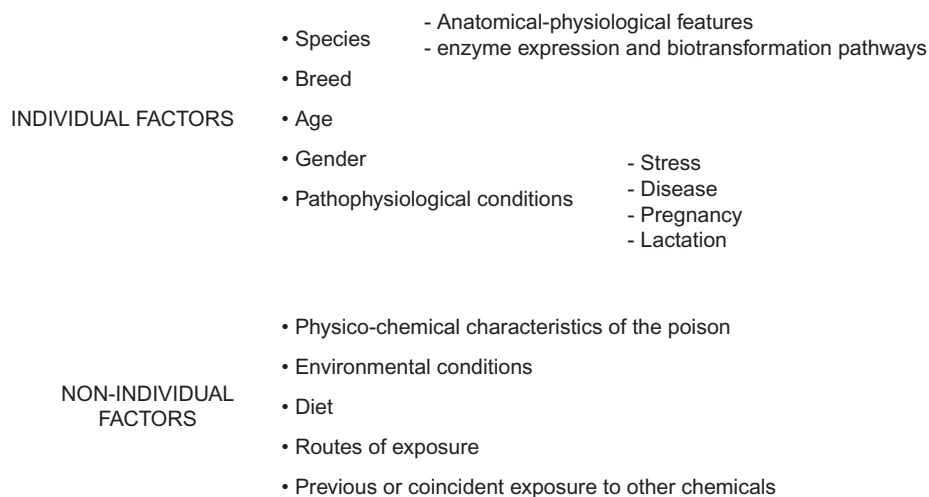
It has long been known that the sensitivity to the adverse effects of xenobiotics is not the same for all species. For example, unlike most mammals, rabbits may ingest large amounts of belladonna (*Atropa belladonna*), an extremely toxic herbaceous plant belonging to the Solanaceae family, without showing significant adverse effects due to the expression of an esterase able to hydrolyze the tropane alkaloid atropine. As a further example, anatomical-physiological features like the substantial inability of vomiting displayed by horses and rats make such species more vulnerable to a wide array of poisons in that, contrary to the other monogastric species, they lack a significant mean of limiting their absorption. On the other hand, the hydrolytic and reductive reactions mainly carried out by the ruminant biota may have opposite effects (i.e., increase vs. decrease of toxicity) according to the nature of chemicals ruminants may be exposed to.

It is also well assessed that the exposure of a population to a given amount of a toxicant may not affect each individual to a similar extent owing to the occurrence of several factors capable of modulating the overall toxic response. Breed may be of importance. Certain shepherd dogs like collies display a high sensitivity to a number of drugs (e.g., avermectins, loperamide and many others) because a significant percentage of such animals do not adequately express certain efflux proteins limiting the drug passage across the blood-brain barrier. Age is also an important determinant of chemical toxicity for most compounds, that is toxicity is generally higher in younger individuals for a number of reasons including, among others, a limited ability in the biotransformation and excretion of xenobiotics. Very little is known as to the

role played by gender and pathophysiological events like starvation, pregnancy and infectious diseases in modulating the response to poisons in veterinary species; stress is generally reported to worsen the clinical picture of several toxic syndromes and even to act as the starter event in the onset of copper toxicosis in sheep.

A number of non-individual conditions, i.e., of environmental origin as well as pertaining to or mediated by the xenobiotic itself, may also influence the response to foreign compounds. As regards the environmental factors, diet composition and notably dietary imbalances may be important determinants, as in the case of sulfur amino acids and antioxidants (e.g., selenium, vitamin E, carotenoids) for a large array of pro-oxidant drugs and toxicants like iron, copper, paraquat or acetaminophen. Even cold or warm atmospheric temperatures may play a role in exacerbating the toxic effects of certain chemicals. For a given poison, toxicity may also vary according to the particle size and the route of exposure. In some instances, certain pesticides, especially some organophosphorus derivatives, may spontaneously give rise to isomers of much higher toxicity (i.e., isomalathion) when stored in organic solvents or water. In addition to the above factors, the previous exposure to a number of chemicals known to respectively decrease (e.g., cimetidine, azole fungicides) or increase (e.g., phenobarbitone, organochlorines) the biotransformation capacity of a living organism may significantly alter the response toward poisons according to the toxicological properties of their metabolites. A telling example is the lower sensitivity to warfarin and other anticoagulant rodenticides of dogs subjected to phenobarbitone treatment for the prophylactic or therapeutic control of convulsive seizures of epilepsy.

Finally, a number of kinetics or dynamics interactions are described in which the concurrent administration of



**FIGURE 4.1** General scheme of the individual and non-individual factors affecting chemical toxicity.

one or more active principles, generally for therapeutic purposes, may displace a drug or a toxicant from its binding to plasma proteins, receptors or from the active site of enzymes involved in its biotransformation, leading to an abrupt increase in the concentration of the displaced compound with often severe and sometimes fatal consequences. The purpose of this chapter is to illustrate the main individual and non-individual factors capable of modulating chemical toxicity in veterinary species. A list of such factors is provided in [Figure 4.1](#).

## INDIVIDUAL FACTORS

### Species

#### *Anatomical and physiological features*

Vomiting is an important physiological function which may be of vital importance in limiting the bioavailability of an ingested toxicant, as often occurs in dogs and cats. In the horse, however, the marked tone at the cardiac sphincter makes vomiting a remarkably seldom event, which often occurs with gastric rupture ([Smith and Magdesian, 2009](#)). The incapability of vomiting displayed by rats has been taken into account to design targeted rodenticides like red squill, which is characterized by emetic properties notably increasing its safety for most domestic species ([Barnett et al., 1949](#)).

The effects of several poisons may vary to a considerable extent in polygastric vs. monogastric species. First of all, the considerable volume of ruminal fluid (100 to 225 L in cattle and 10 to 25 L in sheep and goats) leads to a dilution of toxic principles decreasing their absorption rates. Second, and most importantly, ruminal microorganisms carry out a number of biotransformation reactions, mainly

hydrolytic and reductive in nature, that are responsible for the bioactivation or detoxification of poisonous substances. Common examples for the first event are the hydrolysis of plant cyanogenic glycosides (e.g., dhurrin, amygdalin, etc.) leading to the release of cyanides ([Vetter, 2000](#)) or the reduction of nitrates to nitrites, which are largely implicated in nitrate toxicosis due to their vasodilating and methemoglobin-forming properties lacking in the parent compound ([Bruning-Fann and Kaneene, 1993](#)). On the contrary, both rumen protozoa and bacteria may detoxify many mycotoxins including ochratoxin A, which is hydrolyzed into the non-toxic ochratoxin  $\alpha$  and phenylalanine, and trichothecenes (T2-toxin, diacetoxyscirpenol and deoxynivalenol), mainly via a reductive de-epoxidation and hydrolytic deacetylation giving rise to much less toxic metabolites ([Swanson et al., 1987](#); [Yiannikouris and Jouany, 2002](#)). In addition, a bacterial lactonohydrolase is thought to convert the fusariotoxin zearalenone into the non-estrogenic product 1-(3,5-dihydroxy-phenyl)-10-hydroxy-1-undeca-6-one ([Takahashi-Ando et al., 2002](#)). An overview of the ruminal fate of a number of poisonous chemicals and the outcome of the biotransformation pathway (activation vs. detoxification) is presented in [Table 4.1](#).

Renal clearance of chemicals is largely affected by tubular reabsorption, which takes place by passive diffusion and thus depends on both the concentration of the xenobiotic in tubular fluid and its degree of ionization. The latter, in turn, is determined by the pKa of the compound under consideration and the pH of the tubular fluid, which may be assumed to be that of urine and is therefore influenced by dietary habits. Acidic values (5.5 to 6.9) are related to diets rich in animal proteins characterizing carnivorous species but also suckling or milk-fed individuals (e.g., veal calves), whereas alkaline values (7.2 to 8.4) are found in herbivorous species ([Baggot, 2001](#)). Therefore, it is anticipated that weak organic acids (e.g., paracetamol and many other NSAIDs, ochratoxin A) will



TABLE 4.1 Influence of rumen on the toxicity of selected compounds

Compound	Type of reaction	Metabolite	Outcome
Cyanogenic glycosides	Hydrolysis	Cyanide ions	Bioactivation
Ochratoxin A	Hydrolysis	Ochratoxin $\alpha$	Detoxication
Aflatoxin B1	Reduction	Aflatoxicol	Detoxication
Zearalenone	Hydrolysis	1-(3,5-dihydroxy-phenyl)-10-hydroxy-1-undeca-6-one	Detoxication
T-2 toxin	Hydrolysis (deacetylation)	HT-2 toxin	Detoxication
Urea	Hydrolysis	Ammonium ions	Bioactivation
Nitrates	Reduction	Nitrites	Bioactivation
Organophosphates	Hydrolysis	§	Detoxication
Pyrethroids	Hydrolysis	§	Detoxication
Carbamates	Hydrolysis	§	Detoxication
Nitrophenols	Reduction	Aminophenols	Detoxication

§ Hydrolyzed metabolites of various structure according to the nature of the parent compound

be present mostly in their non-ionized forms and hence efficiently reabsorbed in the presence of acidic urines, and the same holds true for weak organic bases (e.g., alkaloids like strychnine, caffeine or theobromine, and quaternary ammonium compounds) in herbivore urines.

#### Enzyme expression and biotransformation pathways

It is believed that the expression of glucose 6-phosphate dehydrogenase (G6PDH), especially in the erythrocytes, is comparatively lower in sheep with respect to the other species (Maronpot, 1972). This enzyme plays a key role in NADPH supply thus influencing the glutathione (GSH) reductase-mediated regeneration of GSH. Such tripeptide is part of a very effective antioxidant system including selenium-dependent GSH-peroxidase and vitamin E that contrasts hemoglobin oxidation to methemoglobin as well as its denaturation to Heinz bodies. Accordingly, sheep are expected to be particularly susceptible to pro-oxidant chemicals like copper, nitrites and chlorates or to hemolytic principles of vegetable origin like S-methylcysteine sulfoxide. Compared to other species, feline hemoglobin is markedly liable to oxidative damage; this has been attributed to both the presence of eight -SH groups per molecule and the ready dissociation of hemoglobin from the tetramer to dimer form (Harvey and Kaneko, 1976).

The biotransformation of foreign compounds is mainly mediated by hepatic and extrahepatic enzyme systems, collectively known as xenobiotic metabolizing enzymes (XMEs), aimed at increasing the hydrosolubility of the resulting metabolite(s) which may be more easily excreted via the urinary or the biliary routes. Two phases are essentially recognized. During phase I, different polar groups ( $-\text{OH}$ ,  $-\text{NH}_2$ ,  $-\text{COOH}$ ) are introduced or unmasked in the molecule's backbone, which enable them to undergo the subsequent phase II; it is accomplished by several families of cytochrome P450

(CYP)-dependent monooxygenases, different reductases, and enzymes capable of performing hydrolytic reactions called esterases or amidases. Phase II is synthetic in nature, in that a number of endogenous molecules such as acetate, sulfate, glycine, GSH or glucuronic acid are conjugated by their corresponding transferases to the parent molecules and/or to their phase I metabolites. While the conjugated metabolites are invariably of negligible toxicity, phase I metabolites may become more (re)active than the parent compound as a result of a bioactivation process, like for instance in the case of the CYP1A-mediated epoxidation of aflatoxin B1 (AFB1) to AFB1 epoxide or the CYP2B-dependent oxidative desulfuration of diazinon to diazoxon. Taking into account the influence of biotransformation processes on both the residence time and the activation/inactivation rate of a given chemical, it is therefore obvious that species-related overt differences in XME expression and functions may be considered as capital factors dictating the susceptibility to poisons (Nebbia, 2001). As regards phase I enzymes, however, only circumstantial evidence is provided by studies published so far concerning comparative CYP expression in domestic animals (Ioannides, 2006; Fink-Gremmels, 2008). For example, as documented by a very low oral  $\text{LD}_{50}$  (2–3 mg/kg body weight), the horse displays an unusual sensitivity toward monensin and other polyether ionophores antibiotics, which are widely used as coccidiostats and zootechnical additives in other livestock species. This is believed to reflect, among other factors, the relative lower ability of *Equidae*, as compared to many food producing species and the rat, to perform the CYP3A-mediated oxidation of such drugs yielding biologically inactive metabolites (Table 4.2), although further research is needed to confirm whether a comparatively lower CYP3A expression really occurs in the equine species (Nebbia *et al.*, 2001).

Likewise, in farm animals the magnitude of estrogenic effects arising from the exposure to the mycotoxin

TABLE 4.2 Sensitivity to ionophores of horses: relationship between the rate of the *in vitro* cytochrome P450-mediated oxidation of monensin, liver CYP3A content and the oral lethal dose of monensin in different animal species

Species	Oxidative monensin metabolism <sup>a</sup> (nmol · nmol cyt. P450 <sup>-1</sup> )	CYP3A content <sup>a</sup> (arbitrary units)	Monensin lethal dose <sup>b</sup> (mg · kg bw <sup>-1</sup> )
Horse	0.4	61	2–3
Rat	0.7	100	35
Swine	0.8	107	40–60
Cattle	1.5	123	60–80
Chicken	3	167	200

<sup>a</sup>Nebbia *et al.* (2001)

<sup>b</sup>Anadón and Martínez-Larrañaga (1991)

zearalenone correlates well with the extent of the generation of the  $\alpha$ -reduced metabolite  $\alpha$ -zearalenol over the  $\beta$ -stereoisomer cognate  $\beta$ -zearalenol, being maximal in the pig, intermediate in cattle and lowest in chickens. Whether such phenomenon reflects a species-related different expression of  $\alpha$ - or  $\beta$ -hydroxysteroid-dehydrogenases remains to be established (Fink-Gremmels and Malekinejad, 2007).

As to phase II reactions, it is known that the conjugation with GSH is the critical pathway in detoxifying aflatoxin B1 (AFB1)-epoxide (AFBO), a reactive metabolite accounting for both the AFB1-mediated cytotoxicity and the DNA-damaging effects. As detected by the measurement of the GSH-AFBO adducts in liver preparations, the remarkable susceptibility of the turkey to AFB1 has been related to the lack of apparent GSH-conjugation of AFBO, despite the presence of proteins cross-reacting with antibodies to GSH *S*-transferases isoforms (Yc and Yc<sub>2</sub>) performing such reaction in the rat (Klein *et al.*, 2002). Relevant qualitative species-related differences in phase II enzymes are indeed long recognized and some of these have been recently characterized at gene level. Compared to most mammalian species, cats are known to be more sensitive to phenol-containing compounds, including aniline derivatives such as acetaminophen (Court, 2001), many other NSAIDs, pyrethroids and a number of organophosphorus and carbamate derivatives. This feature, also shared by other *Felidae* and the domestic ferret (Court, 2001), has been linked to a structural defect of a gene encoding for the UGT (uridine diphosphoglucuronyltransferase) 1A6 enzyme giving rise to a non-functional protein (Court and Greenblatt, 2000). Another example involves the N-acetylation of arylamine drugs (e.g., *p*-aminophenol, sulfonamides), an important detoxification pathway catalyzed in most mammals by at least two N-acetyltransferases (NAT-1 and NAT-2). Poor N-acetylating capacity is found in cats (and in other *Felidae*) due to the lack of NAT-2 gene (Trepanier *et al.*, 1998), while the absence of both genes does not allow this reaction to occur at all in dogs and *Canidae* (Trepanier

*et al.*, 1997). An unusual sensitivity to the prolonged therapies with sulfonamides is commonly reported in dogs, and consists mainly of fever, arthropathy, blood dyscrasias (neutropenia, thrombocytopenia, or hemolytic anemia), cholestasis or hepatic necrosis, skin eruptions, uveitis, or keratoconjunctivitis sicca. The inability of dogs to carry out the N-acetylation of the arylamine derivatives causes the sulfonamides to be oxidized by CYPs and even by myeloperoxidases to hydroxylamines, which in turn may be bioactivated to reactive nitroso metabolites responsible for both cytotoxicity and hapten formation (Lavergne and Trepanier, 2007). More recently, the deficient N-acetylating capacity displayed by domestic carnivores has been implicated in the hematotoxicity characterizing the acetaminophen exposure, in that the *p*-aminophenol resulting from the carboxylesterase-mediated hydrolysis of the drug can no longer be acetylated back to the parent molecule and is therefore free to oxidize hemoglobin to methemoglobin in erythrocytes from exposed cats and dogs (McConkey *et al.*, 2009).

## Breed

The influence of breed in affecting the susceptibility to a number of drugs and toxicants has been identified for many years in domestic animals. For instance, Brahman cattle have been reported to be more sensitive to some organophosphates compared to other European breeds for still unknown reasons (Randell and Bradley, 1980). As a further example, the very low percent body fat characterizing greyhound dogs is likely to result in lower than expected volume of distribution and hence in higher serum drug concentrations as compared to that seen in breeds with higher percent body fat. This feature put the greyhounds and all “thin” dog breeds at higher risk for developing toxicity following the exposure to highly lipophilic compounds such as organochlorine or organophosphorus pesticides, and possibly many others (Fleischer *et al.*, 2008). In recent years, the rapid advancement of molecular techniques has allowed us to conclude that most of the observed population-based differences in sensitivity to drugs and toxicants is genetically determined and is almost invariably related to variation in XMEs and other proteins involved in drug kinetics (Mealey, 2006). Polymorphisms are defined as genetic variations occurring at a frequency of at least 1% in a given population. There is scant information in veterinary species about genetic polymorphisms relevant to the purposes of this chapter and most of the published literature is concerned with the canine species (Fleischer *et al.*, 2008). For example, both CYP2B liver expression and the related *in vitro* ability to oxidize the anesthetic compound propofol to its pharmacologically inactive metabolite 4-hydroxypropofol were remarkably lower in greyhounds

TABLE 4.3 Selected P-glycoprotein substrates

Class	Compounds	Class	Compounds
<i>Anticancer agents</i>	Doxorubicin	<i>Opioids</i>	Loperamide
	Docetaxel*		Morphine
	Vincristine*		Butorphanol
	Vinblastine*	<i>Cardiac drugs</i>	Digoxin
	Etoposide*		Diltiazem*
	Taxol		Verapamil*
<i>Steroid hormones</i>	Actinomycin D		Talinolol
	Aldosterone	<i>Immunosuppressants</i>	Cyclosporin
	Cortisol*		Tacrolimus
	Dexamethasone*		Ivermectin*
<i>Antimicrobials</i>	Methylprednisolone*	<i>Others</i>	Other avermectins
	Erythromycin*		Amitriptyline
	Ketoconazole		Terfenadine*
	Itraconazole*		Ondansetron
	Tetracycline		Domperidon
	Doxycycline		Acetpromazine
	Levofloxacin		Monensin*
	Sparfloxacin		

Data derived from Gombar *et al.* (2004); Mealey (2006); Martinez *et al.* (2008)

\*Also CYP3A substrates

compared to beagle dogs. Taking also into account the consequences of the poor ability to store lipophilic compounds in fat depots mentioned above, these findings indicate that in greyhounds an adjustment of the propofol dosing regimen is required to avoid delayed recovery and other untoward effects (Hay Kraus *et al.*, 2000). While the specific genetic alteration responsible for the low CYP2B expression in such dog breeds is yet unknown, a true genetic polymorphism has been demonstrated for the gene encoding for thiopurine S-methyltransferase (TPMT), a phase II enzyme carrying out the detoxification of thiopurine drugs (e.g., azathioprine), which are widely used in humans and in companion animals to treat neoplasia and as immunosuppressants. A wide range of TPMT red blood cells activity was found in different breeds of dogs, with a nine-fold variation in median values between the giant schnauzer (lowest activities) and the Alaskan malamute (highest activities) (Kidd *et al.*, 2004). An almost six-fold variation in TPMT red blood cells activity was detected in 104 cats of unspecified breed, which was largely attributable to a TPMT gene polymorphism (Salavaggione *et al.*, 2004); the limited extent of thiopurine drugs inactivation occurring in low TPMT individuals results in life-threatening toxicity, most often myelosuppression (Rodriguez *et al.*, 2004).

Other well-defined genetic polymorphisms involving P-glycoproteins (P-gps) have been documented in dogs and are under investigation in other veterinary species like sheep, cattle and chickens (Mealey, 2006; Martinez *et al.*, 2008). P-gps are ATP-dependent pumps encoded by the MDR-1 (now ABCB1) gene; they belong to the

family of drug transporters and are expressed in a variety of tissues, i.e., the enteric epithelium, the brain capillary endothelial cells, the bile canaliculi, the renal tubular epithelial cells, the placenta, the ovary and the testes, where they catch selected xenobiotics (defined as “substrates”) and either extrude them out of the body (i.e., into the bile, the urine or the intestinal lumen) or prevent them from accessing certain vital sites (Gombar *et al.*, 2004). A list of such substrates is provided in Table 4.3.

In so doing, they play a key role together with XMEs in governing the absorption, the distribution and the excretion of xenobiotics, and a unique role in protecting the brain (blood–brain barrier), the genital organs and the fetus. An intriguing characteristic of such carriers is that a number of their substrates are also CYP3A substrates (Martinez *et al.*, 2008). The MDR-1 polymorphism in dogs is common in herding breeds (collies, old English sheepdogs, Australian shepherds, English shepherds, German shepherds, border collies, long-haired whippets, silken windhounds) and is based on a four base-pair deletion mutation causing a shift in the reading frame generating, in turn, a number of premature stop codons; in addition to the wild-type MDR1 (+/+), dogs may be homozygous, i.e., MDR1(–/–), or heterozygous, i.e., MDR1(+/–) for the mutation (Mealey *et al.*, 2001). A very high allelic frequency of MDR1 mutation has been detected in dogs, being as high as 55% in collies, 42% in long-haired whippets, and about 20% in Australian shepherds (Mealey, 2006). A moderate to sharp increase in both the overall bioavailability and residence time of P-gp substrates along with a wide array of adverse effects are therefore expected

in MDR(+/-) and, to a higher extent, in MDR(-/-) dogs, respectively. In such animals, macrocyclic lactones widely used as antiparasitic agents, such as ivermectin, moxidectin, milbemycin, and selamectin, are no longer, or only to a limited extent, prevented from crossing the blood-brain barrier and cause neurotoxicity (hypersalivation, mydriasis, ataxia, muscle tremors, depression and coma) following the exposure to therapeutic doses. A long-lasting central nervous system depression has been described in MDR(-/-) dogs treated with either the phenothiazine derivative acepromazine or the morphine derivative butorphanol (Mealey, 2006) and a similar syndrome has been observed in MDR1-mutant dogs orally administered with loperamide, a synthetic opioid used to control intestinal motility (Sartor *et al.*, 2004); all such drugs are characterized by a wide safety margin in wild-type individuals, in which they fail to enter the brain tissue. On the other hand, myelosuppression and gastroenteric disturbances have been reported in dogs homozygous for MDR-1 mutation subjected to anticancer therapy with doxorubicin or vincristine, and such severe side effects have been linked to a reduced rate of drug renal and/or biliary excretion (Mealey, 2006). Finally, a homozygous genomic deletion encompassing exon 2 of the COMMD1 gene playing a critical role in copper biliary excretion has been identified as the genetic cause of the canine copper toxicosis showing a high prevalence in Bedlington terriers (De Bie *et al.*, 2005) and closely resembling the Wilson disease described in humans.

## Age

It is generally accepted that sensitivity to the effects of drugs and other foreign chemicals is often greater in young and elderly individuals than in adult ones as the result of several factors including differences in the rate of absorption, binding to plasma proteins, distribution, biotransformation and excretion (Baggot, 2001). In newborn and lactating animals, the combined effects of a lower hepatic biotransformation rate and a less efficient renal excretion considerably decrease the rate of elimination of foreign chemicals. Although some species-related differences are reported to occur, it may be assumed that, in the majority of mammals aging about 12 weeks, both functions correspond to or approach those of adult individuals (Baggot, 2001; Hines, 2008). Another factor characterizing lactating individuals is the acidic urinary pH influencing the tubular reabsorption of weakly acidic xenobiotics (see above); in young ruminants, moreover, the lack of a functional rumen may also be of importance in affecting the overall outcome of the exposure to many toxicants. Accordingly, calves are reported to be particularly susceptible to a number of mycotoxins or to pesticides like organophosphates, carbamates, or pyrethroids,

which undergo a considerable breakdown in the ruminal environment. Theoretically, the reduced rate of CYP-mediated oxidations should lead to a parallel decrease in the generation of (re)active metabolites rendering young individuals less susceptible to toxicants requiring CYP bioactivation (e.g., thionophosphates, acetaminophen, aflatoxin B1, ipomeanol). However, it should be stressed that the final outcome of the exposure to any poison is dictated by the balance between bioactivating vs. detoxicating pathways, the latter being almost invariably performed by conjugating enzymes. Accordingly, the much higher sensitivity to AFB1 displayed by turkey poults with respect to adult ones is related to the remarkably lower expression of the GST isoenzyme Yc bearing a high affinity toward the CYP-mediated AFB1 epoxide (AFBO) (Klein *et al.*, 2002) and similar mechanisms are likely to occur in piglets (Clarkson, 1980) and chicks (Quezada *et al.*, 2000). With the likely exceptions of sheep (Kaddouri *et al.*, 1990) and goats (Eltom *et al.*, 1993), in which glucuronidating capacity would be already well expressed in the first weeks of life, the low expression of UGTs is considered one of the crucial determinants of the increased susceptibility of young animals to several poisons (Hines, 2008). As an example, while a low to moderate zearalenone contamination of feedstuffs often goes unnoticed in sows under field conditions, the poor glucuronidation rate of both zearalenone and its metabolites  $\alpha$ -zearalenol and  $\beta$ -zearalenol, which are known to cross both the placental and the mammary barriers, accounts for the marked susceptibility of newborn piglets to the estrogenic effects of such compounds (Dacasto *et al.*, 1995). The enteric absorption of certain toxicants may be enhanced in the early age. Possibly related to a high pinocytotic activity of the immature epithelium, an increased oral bioavailability has been demonstrated for a number of metals (lead, cadmium, iron, cobalt, zinc and mercury) in newborn or young individuals (Nordberg *et al.*, 2007), reaching up to 90% in the case of lead (Humphreys, 1991).

Finally, there is meager information concerning the effects of elderly on the sensitivity to poisons in domestic animals, and notably in food producing species because of the short duration of their life cycle. Impaired hepatic and renal xenobiotic clearance are described in elderly people, mainly due to reduced blood flow and glomerular filtration rate rather than to a significant decrease in the biotransformation capacity (Hilmer, 2008). Likewise, limited differences in the activity of hepatic phase I and phase II XME have been detected in adult vs. aged individuals in sheep (Kaddouri *et al.*, 1990) or dogs (Kawalek and El Said, 1990), while the expression and the activity of selected liver CYPs, hydrolytic and conjugative enzymes have been even reported to significantly increase in mares aged more than 12 years compared to those in the age range 1 to 2.5 years (Nebbia *et al.*, 2004).



## Pathophysiological conditions

### Stress

In sheep, the abrupt release of copper in the bloodstream starting the hemolytic crisis which characterizes the onset of copper toxicosis is triggered by stressful events like shearing or transhumance (Ortolani *et al.*, 2004); similarly, mild to moderate stressing conditions like loud noise, bright light and touch may provoke a fatal crisis in strychnine poisoned dogs (Talcott, 2006). Prolonged food and water deprivation may increase the toxicity of many drugs by reducing the rate of their elimination and lowering the concentration of certain antioxidants such as GSH (Bidlack *et al.*, 1986). An increase in the parent drug mean residence time and a reduced amount of both plasma glucuronide and sulfate derivatives were recorded in either food-withheld or water-deprived calves dosed with paracetamol, pointing to an inhibition of both UGTs and sulfotransferases (Janus *et al.*, 2003).

### Disease

It is generally accepted that diseased animals are less able to eliminate xenobiotics as the result of both a direct damage of organs and tissues involved in the biotransformation reactions and the alterations in protein binding, cofactor availability and renal clearance (Baggot, 2001). The complex events of the so-called “acute phase reaction” following the exposure to microbes or parasites include the release of several pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF) $\alpha$ . Such cytokines are responsible for both an overall reduction in the biotransformation capacity remarkably affecting CYP-dependent monooxygenases and UGTs in liver but also in extrahepatic tissues (Galtier and Alvinerie, 1996; Monshouwer and Witkamp, 2000), as well as of the inhibition of P-gps and of several other drug transporters (Petrovic *et al.*, 2007; Martinez *et al.*, 2008). Moreover, the synthesis of a number of acute phase proteins such as fibrinogen, haptoglobin and C-reactive protein, and the consequent alteration in the plasma protein profile, may result in an increase in the amount of unbound xenobiotics which are free to distribute in tissues and exert their pharmacological or toxicological action(s) (Mills *et al.*, 1997). Pre-existing kidney damage, as may commonly occur in aged carnivores, may also generally lower the rate of excretion of most chemicals and increase the toxicity of nephrotoxics such as ethylene glycole or melamine.

### Pregnancy

Little is known concerning the effects of pregnancy on chemical toxicity in veterinary species. The numerous physiological changes occurring in pregnant animals, especially concerning hormone secretion, increase in

plasma volume, decrease in plasma albumin concentration and increase in body fat (Martinez and Modric, 2010), are expected to considerably alter xenobiotic kinetics, but the underlying mechanisms still remain largely unknown. Plasma concentrations of steroid hormones, mainly estrogens and progesterone but also cortisol, are known to rise steadily during pregnancy and to activate some nuclear receptors (e.g., estrogen receptor  $\alpha$ , constitutive androstane receptor) resulting in a complex modulation of several XMEs. In pregnant women, a number of enzymes are known to be up-regulated (CYP2A6, 2D6, 2C9, 3A4 and UGT1A4), while others (CYP1A2 and CYP2C19) are reported to be depressed; consequently, the *in vivo* clearance of a number of drugs may be increased (e.g., nicotine, phenytoin, glyburide, etc.) or decreased (e.g., caffeine, theophylline) (Jeong, 2010). As marked differences across species are known, even between laboratory rodents, the overall effects of pregnancy on xenobiotic kinetics are difficult to predict in veterinary species, including dogs and cats (Rebuelto and Loza, 2010).

### Lactation

Lactation is reported to increase the clearance and the volume of distribution of many drugs (Martinez and Modric, 2010). Lactating animals may also excrete significant amounts of poisons through the mammary gland. In the case of highly lipophilic substances like polychlorodibenzodioxins/furans (PCDDs/PCDFs) and polychlorobiphenyls (PCBs), this implies on the one hand a significant reduction of the dam body burden through such additional way of excretion (Rychen *et al.*, 2008) but poses potentially toxic risks to the suckling offspring. In this respect, experimental studies conducted in goats and cattle have demonstrated that greater quantities of PCDDs, PCDFs or PCBs are transferred via suckling to neonates than through the placenta to fetuses (Lyche *et al.*, 2004; Hirako, 2008). Alterations of the plasma gonadotropins profile, decrease in plasma testosterone levels and increase in sperm DNA damage were recorded in goat kids exposed to PCB153 both during the second half of gestation and through lactation until weaning, which takes place about 40 days after parturition (Oskam *et al.*, 2005). According to Panter and James (1990), many plant toxins may be excreted in milk and cause toxicosis in suckling ruminants and foals. This is the case for tremetol and tremetone from white snakeroot (*Eupatorium rugosum*) or rayless goldenrod (*Haplopappus heterophyllus*) inducing the so-called “milk fever” characterized by severe muscle, myocardial and liver damage, or for the hepatotoxic pyrrolizidine alkaloids mainly found in the Boraginaceae (*Heliotropium* spp.), Leguminosae (*Crotalaria* spp.) and Compositae (*Senecio* spp.) families. Glucosinolates are glycosides found in many crucifers of genus *Brassica* that are important for animal nutrition,



such as rapes, kales, cabbages, turnips and others; upon hydrolysis, glucosinolates yield a number of derivatives including goitrin, thiocyanates and isothiocyanates, capable of depressing thyroid function. Reduced growth rate and lowered serum thyroxin levels were found at weaning in kids fed on milk from goats administered defatted meal of meadowfoam (*Limnanthes alba*), a glucosinolate-containing Limnaceae (White and Cheeke, 1983). There is also evidence that cyanides released from cyanogenic glycosides as well as their metabolites thiocyanates may also be transferred to milk and be responsible for a chronic syndrome in suckling goat kids associated with histological changes in brain, thyroid, liver and kidney (Soto-Blanco and Górniak, 2003). In some instances, according to the amount and/or the nature of toxic chemicals excreted through the mammary gland, the contaminated milk may be the cause of acute toxicosis. The documented excretion of zearalenone and its metabolites  $\alpha$ -zearalenol and  $\beta$ -zearalenol in porcine milk (Fink-Gremmels and Malekinejad, 2007) has been associated with the onset of a severe hyperestrogenic syndrome in suckling female piglets (Dacasto *et al.*, 1995). As a further example, cats may become acutely poisoned by metaldehyde by ingesting milk from an intoxicated cow (Gennaro Soffietti and Nebbia, 1984).

## NON-INDIVIDUAL FACTORS

### Physico-chemical characteristics of the poison

The physical state of the toxicant is of importance. Fine particles are more readily absorbed than coarse ones; in the case of poisons bearing irritating properties (e.g.,  $\alpha$ -naphthylthiourea, zinc phosphide), however, small size particles come into contact with a wider surface of the gastric mucosa and hence may more likely elicit protective vomiting.

When using commercial preparations of pesticides, it is always recommended to follow the datasheets and the instructions for safe use and disposal, avoiding the use of out-of-date products, solutions or emulsions. Such precaution is mandatory for certain organophosphorus insecticides for which storage activation is described with the subsequent spontaneous formation of highly toxic isomers; this phenomenon, which is increased by heat, is reported to continue when the pesticide is diluted and stored. Diazinon, a widely used phosphorothioate insecticide supplied as an emulsifiable dip concentrate for the fight against a wide array of ectoparasites in ruminants, has been involved in a toxic outbreak in which 81 of a group of 210 lambs died within 12 hours after being dipped with the contents of a partly used container of 15-year-old sheep dip. Analysis of dip samples revealed

the presence of a number of highly toxic spontaneous hydrolysis breakdown products of diazinon, including TEPP, sulfoTEPP and the oxon derivatives of diazinon and TEPP (Sharpe *et al.*, 2006). In addition, trichlorophon, which is sold as a wettable powder to control external parasites of cats and dogs, may be easily converted in alkaline solutions to dichlorvos, which is characterized by an eight-fold lower oral LD<sub>50</sub> in rats compared to the parent compound (Fonnum and Lock, 2000).

Solvents and other substances included in commercial preparations may also affect the overall toxicity of the active principle(s). Non-polar solvents may considerably increase the absorption rate of lipophilic poisons, especially when considering the exposure by the dermal route. Piperonyl butoxide (see below) is added to many carbamate- or pyrethroid-based formulations to lower the CYP-mediated detoxification of such compounds thus increasing their insecticidal potency. In other instances, molecules other than the active principle(s) are responsible for some (if not most) of the recorded adverse effects. This is the case of the herbicide Roundup®, a commercial formulation containing 12–50% glyphosate, an organophosphate displaying no anticholinesterase activity, and very low bioavailability and toxicity (oral LD<sub>50</sub> >3500 mg/kg body weight in both goats and rats). The toxic syndrome resulting from the ingestion of recently treated grass and vegetation, including signs of gastrointestinal irritation, swelling of lips and nervous depression, is largely attributable to polyoxyethylene amine, a surfactant used to enhance the penetration of the waxy surfaces of plants by the active ingredients, which has an oral LD<sub>50</sub> in rats of about one third that of glyphosate (Campbell and Chapman, 2000).

### Environmental conditions

High ambient temperatures are reported to enhance the toxicity of chlorophenols and nitrophenols that cause an increased production of heat by uncoupling mitochondrial oxidative phosphorylation (Fikes *et al.*, 1989). Conversely, cold temperatures are predisposing factors for  $\alpha$ -chloralose, a rodenticide/avicide formerly used as an anesthetic agent, which may induce a life-threatening hypothermia especially in poisoned cats by acting on hypothalamic thermo-receptors (Segev *et al.*, 2006).

### Diet

Poor or unbalanced diets, particularly when deficient in certain trace elements or in antioxidants, may significantly increase the dangerousness of a number of toxicants. Diets low in sulfur and molybdenum are reported to increase the susceptibility of sheep to copper, due to

the ability of such elements to promote the formation of relatively insoluble complexes limiting copper bioavailability (Nederbragt *et al.*, 1984). Likewise, copper-deficient ruminants are more prone to develop molybdenosis, a syndrome characterized by scouring, weight loss, hair depigmentation, reproductive impairment and even death (Raisbeck *et al.*, 2006). A primary vitamin K deficiency seldom occurs, but prolonged oral or parenteral therapies with certain antibacterial drugs may suppress the enteric flora resulting in a lack of vitamin K synthesis predisposing dogs and other non-target species to anticoagulant rodenticides toxicity (Prater, 2006).

Diets should also ensure a proper supply of sulfur amino acids, which participate in the control of oxidative status due to their involvement in the synthesis of GSH in tissues and erythrocytes (Tesseraud *et al.*, 2009), and of other components of the antioxidant systems including, among others, selenium, as cofactor of GSH-peroxidase (Se-GSHPx), vitamin E and ascorbic acid. Dietary deficiencies in one or more antioxidants are important risk factors for toxicants being biotransformed to reactive metabolite(s) or generating reactive oxygen species. Low GSH levels have been implied as a major condition in enhancing the liver toxicity of acetaminophen in cats and dogs, due to the consequent reduced ability to form nontoxic GSH-adducts with the CYP2E1-mediated reactive imino-derivative (NAPQI, i.e., N-acetyl-*p*-benzoquinoneimine) of the analgesic drug (Savides *et al.*, 1985). In this respect, the oral administration of bioflavonoid antioxidants to cats elicited a beneficial effect on their ability to resist acetaminophen-mediated oxidative injury to erythrocytes (Allison *et al.*, 2000). Iron oral or parenteral supplementation to piglets represents a common practice in piggeries to prevent neonatal anemia. Piglets from sows fed diets marginal in vitamin E or rich in polyunsaturated fatty acids, which may cause a secondary vitamin E deficiency due to the relative ease of undergoing peroxidation, are much more susceptible to develop lethal toxicoses with routine iron treatments than piglets nursing dams fed a vitamin E-supplemented diet (Lipinski *et al.*, 2010). On the other hand, selenium but not vitamin E supplementation to chicks was able to reduce the lethality of paraquat, a dipyrldyl-herbicide whose toxicity is based on redox cycling and intracellular oxidative stress generation (Combs and Peterson, 1983).

## Routes of exposure

For most xenobiotics, parenteral routes of exposure entail a more prompt and complete bioavailability than the oral one and therefore often result in a lower LD<sub>50</sub>. In addition, orally ingested poisons may undergo a first pass metabolism that in certain cases may lead to an almost complete detoxification. As an example,

pyrethrum-based insecticides are practically nontoxic in mammalian species, in which they are extensively hydrolyzed in the gut, but not in fish, where a significant amount of the molecule may enter the body through the gills. As a consequence, while the oral LD<sub>50</sub> of pyrethrin in the rat amounts to 1500 mg/kg body weight, it is as low as 5.2 µg/L in the trout (*Onchorhynchus mykiss*) (Anadón *et al.*, (2009)).

## Previous or coincident exposure to other chemicals (drug–drug interactions)

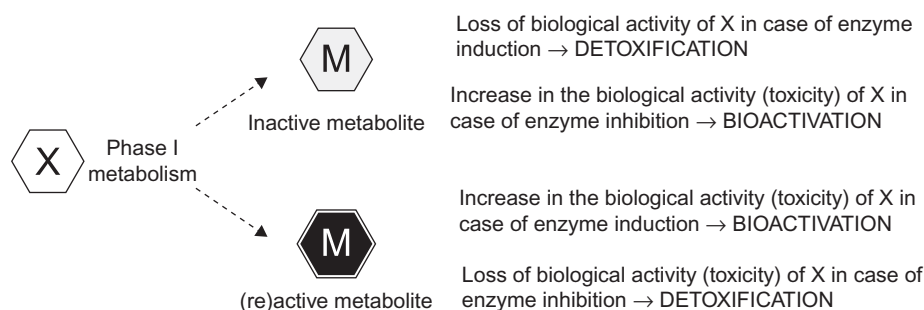
A variety of chemicals (drugs, plant toxins, pesticides, environmental pollutants) animals may be exposed to are capable of increasing (enzyme inducers) or decreasing (enzyme inhibitors) the expression and the activity of hepatic and extrahepatic phase I and phase II enzyme systems participating in the biotransformation reactions hence modulating the toxicity of several xenobiotics. A detailed description of the nature of inducers or inhibitors and the mechanisms by which they affect XMEs is beyond the scope of this chapter (for a review see Lin and Lu, 1998; Handschin and Meyer, 2003). As regards enzyme induction, suffice it to say that generally this phenomenon takes place slowly as it requires a prolonged exposure to the inducing molecule(s). Phase I enzymes and most notably CYPs are more inducible than conjugating enzymes, with the remarkable exception of UGTs and GSTs. More to the point, while inducers like phenobarbitone (PB), organochlorine insecticides or PCDDs are able to affect both phase I and inducible phase II enzymes, others are much more selective, as is the case of rifampicin or certain macrolides known to induce CYP3A, or of short chain alcohols which are reported to induce CYP2E1. Conversely, chemical-mediated inhibition may occur even after a single exposure and generally involves a single enzyme, which is almost invariably a CYP. The most common forms of inhibition are the competition between two or more chemicals for the same CYP and the formation of complexes between certain molecules (e.g., macrolides, tiamulin, piperonyl butoxide, ketoconazole and other azole fungicides) and a given CYP (in most cases CYP3A). A list of the main CYPs involved in xenobiotic biotransformations, with the most important inducers and inhibitors, is depicted in Table 4.4. For a given toxicant, the modulation of the biotransformation capacity mentioned above may have different clinically relevant outcomes according to the nature of the toxicant under consideration and of its resulting metabolite(s) (Figure 4.2).

In the case of enzyme induction, a decrease of the overall toxicity is expected if the parent compound itself is responsible for the toxic action(s), while the opposite holds true whether one or more (re)active metabolites arising from biotransformation reactions mediate(s)

**TABLE 4.4** List of the main substrates, inducers and inhibitors of CYPs involved in xenobiotic biotransformation in veterinary species

CYP	Substrates	Inducers	Inhibitors
1A	Large planar molecules (e.g., benzopyrene, benzanthracene, polychlorodibenzodioxins, co-planar polychlorobiphenyls, aflatoxin B1), caffeine, theophylline	Polychlorodibenzodioxins, polycyclic aromatic hydrocarbons	Furafylline, $\alpha$ -naphthophlavone, enrofloxacin (?)
2B	Barbiturates, anesthetics (e.g., propofol, ketamine, midazolam) phosphorothioates (e.g., diazinon)	Phenobarbitone	Chloramphenicol, medetomidine, atipamezole
2C	4-Hydroxycoumarin derivatives (e.g., warfarin), tolbutamide, NSAIDs (e.g., naproxen, ibuprofen, diclofenac), diazepam	Phenobarbitone	Sulphaphenazole
2D	Bufuralol, debrisoquine, celecoxib	–	Quinine, quinidine
2E	Small molecules (e.g., alcohols), acetaminophen, acetylsalicylates, anesthetics (e.g., methoxyflurane)	Ethanol	Disulfiram
3A	Most drugs, sexual steroids	Dexamethasone, rifampicin	Tiamulin, erythromycin, triacetyloleandomycin, ketoconazole

Data derived from Lin and Lu (1998); Nebbia (2001); Ioannides (2006); Fink-Gremmels (2008)

**FIGURE 4.2** Consequences of the modulation of the activity of biotransformation enzymes on the toxicity of a given xenobiotic according to the activity of its metabolite(s).

the toxic damage. Although enzyme induction has the potential to modulate the toxicity of a large number of xenobiotics, relatively few examples concerning veterinary species are quoted in the literature. In clinical practice PB is one of the most commonly employed drugs to prevent the onset of seizures in dogs affected by idiopathic epilepsy. One of the most known side effects of PB therapy is the induction of a number of phase I and phase II XMEs, particularly of CYP2B11, CYP2C21 and CYP3A12, which may last up to 4 weeks after the barbiturate is discontinued (Fukunaga *et al.*, 2009). It is anticipated that PB-induced dogs (and probably individuals from other species) are less susceptible to warfarin and possibly other anticoagulant rodenticides (Fink-Gremmels, 2008), pyrethroids (Anadón *et al.*, 2009) and even strychnine (Hatch, 1982a) due to the relatively low toxicity of phase I and phase II metabolites. Little is known about the opposite situation, such as if a bioactivation reaction occurs. To fully acquire

their anticholinesterase activity, for example, insecticidal phosphorothioates (e.g., parathion, diazinon, bromophos, fenthion) require desulfuration to their corresponding oxons, which is believed to be primarily mediated by CYP2B (Buratti *et al.*, 2003), a CYP family reported to be induced by PB and organochlorines (Fink-Gremmels, 2008). Accordingly, the pre-treatment of calves with a single dose of 10mg/kg dieldrin or 10mg/kg PB per day for 21 days increased the toxicity of diazinon as reflected by both the development of more severe clinical signs and a greater depression in whole blood cholinesterase activity with respect to non-induced calves. Interestingly, since the pre-treatment brought about a concurrent increase in the hepatic microsomal activity of both CYP-dependent monooxygenases and of carboxylesterases, it may be concluded that the effects of PB or dieldrin induction were more prominent on the bioactivating (i.e., oxidative) pathway rather than on the detoxifying (i.e., hydrolytic) one

(Abdelsalam and Ford, 1986). Other examples related to the effects of XME inducers on xenobiotic toxicity may be found elsewhere (Nebbia, 2001; Graham and Lake, 2008).

Opposite to enzyme induction, enzyme inhibition is expected to entail beneficial effects in poisonings from toxicants that necessitate a metabolic activation; in certain cases, inhibitors may be used for therapeutic purposes. Cimetidine is recommended to treat acetaminophen toxicosis in dogs and cats because of its inhibitory effects on CYP-mediated generation of the reactive metabolite NAPQI (Fooshee Grace, 2006), which is responsible for the severe liver injury occurring in poisoned animals. As a further example, dogs poisoned by the antifreeze ethylene glycol are treated with 4-methylpyrazole owing to its inhibitory properties toward alcohol dehydrogenase, a cytosolic enzyme catalyzing the oxidation of the parent compound to several metabolites, including glycoaldehyde, glycolic and glyoxylic acids, and oxalic acid, which play a key role in the pathogenesis of ethylene glycol intoxication (Connally *et al.*, 2010). By contrast, enzyme inhibition may enhance the toxic potency of chemicals acting *per se*, i.e., not requiring a metabolic activation, and provides a rationale for explaining many drug–drug interactions also in veterinary species (Fink-Gremmels, 2008). As mentioned before, the most common event is the concurrent administration of (or exposure to) at least two compounds being metabolized by the same CYP(s); in such cases the one showing the greater affinity toward the enzyme involved in its metabolism or being present at higher concentrations may displace the other(s) which remain unmetabolized. Monensin and other ionophores, used worldwide as coccidiostats and zootechnical additives in food producing species, are mainly biotransformed by CYP3A (Nebbia *et al.*, 1999, 2001), the CYP family mostly involved in the metabolism of medicinal substances, yielding oxidized metabolites almost devoid of biological activity (Donoho, 1984). A concomitant administration with drugs displaying a much greater affinity toward CYP3A, such as tiamulin (Nebbia *et al.*, 1999) or macrolides, will cause the unmetabolized ionophores to build up and elicit a severe toxic syndrome in pigs, cattle and avian species involving myocardium, skeletal muscles and the central nervous system (Langston *et al.*, 1985; Szucs *et al.*, 2004). It should not be overlooked that, under conditions of repeated exposure, a number of macrolides (e.g., erythromycin, triacetyloleandomycin, spiramycin) and tiamulin are characterized by a further inhibition mechanism. This involves a CYP3A-mediated N-dealkylation followed by a further oxidation to a nitroso group that binds tightly to the ferrous heme of CYP to form both *in vitro* and *in vivo* a catalytically inactive stable complex without destroying the hemoprotein but slowing down considerably its turnover. Such mechanism has been demonstrated under *in vitro* conditions

in ruminants (Zweers-Zeilmaier *et al.*, 1999) and either under *in vitro* (Carletti *et al.*, 2003) or *in vivo* conditions in rabbits (Cantiello *et al.*, 2003).

The previous exposure to drugs and chemicals may also modulate the expression of P-gps and other drug transporters in veterinary species (Martinez *et al.*, 2008), but relatively few examples of practical relevance are quoted in the open literature. Ketoconazole, a broad spectrum orally active antifungal agent extensively used in veterinary medicine, is reported to inhibit both CYP3A and the P-gps in a variety of animal species including humans (Lin, 2007). The co-administration of ketoconazole and ivermectin to dogs induced both higher plasma concentrations and longer residence times of the latter, with the potential for neurotoxic adverse effects especially for animals displaying genetic polymorphisms of the MDR1 gene (Hugnet *et al.*, 2007).

Drug–drug interactions may also occur in the bloodstream. Anticoagulant rodenticides like warfarin, bromadiolone or brodifacoum are characterized by relatively long plasma half-lives due to a high degree of plasma protein binding. In many cases of anticoagulant rodenticide toxicity, the only clinical signs observed in dogs are rather non-specific (weakness, lethargy or anorexia); in addition, affected animals often display respiratory distress prompting therapeutic intervention. The concurrent administration of anti-inflammatory agents like acetylsalicylates and phenylbutazone or of sulfonamides may cause such drugs to displace the anticoagulant rodenticides from their albumin binding often leading to an abrupt worsening of the clinical picture (Murphy, 2002).

Finally, the co-administration of certain drugs may also modulate the toxicity of several poisons by acting at the receptor level. For instance, drugs that have neuromuscular blocking properties which are elicited through their action on nicotinic receptors (e.g., d-tubocurarine or succinylcholine) may enhance the toxicity of organophosphates or carbamates, while the reverse holds true in the case of the exposure to parasympatholytic agents like atropine, which is widely used as an antagonist in clinical practice (Hatch, 1982b).

## CONCLUSION

The exposure to poisons may elicit different outcomes according to a number of individual and non-individual factors. In most cases the variation in the toxic response is based on the different expression of enzymes or proteins involved in the kinetics of poisons, which may vary according to diet, species, breed and physiopathological factors, or the previous or concomitant exposure to several foreign compounds. While until recently such events could hardly



be explained, currently the unprecedented development of molecular techniques has made it possible to gain insight into many of the mechanisms underlying most of the differences in the response to foreign compounds.

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# Toxicological testing: *in vivo* and *in vitro* models

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## INTRODUCTION

The large number of new chemical entities in human and veterinary pharmaceutical industry has led to increased demand for safety screening to ensure successful drug development. Part of this screening process includes the determination of the toxic potential of these new compounds by applying internationally recognized *in vivo* or *in vitro* toxicological tests. The importance of toxicological testing is critical, as many pharmaceutical and agrochemical products have to be assessed for their safety, before they become available for general use. Furthermore, in some cases, basic research can produce data that eventually leads to the ban of certain chemicals because they were proved to be unsafe.

Animal studies and validated *in vitro* models are extensively used for screening of agents in order to identify and predict potential ill-effects to humans, domestic and farm animals. Although the detection of adverse health effects of xenobiotics is the main objective of toxicity testing, it can be complemented by more sophisticated biomolecular approaches aimed at the elucidation of the mechanisms of action of certain chemicals.

Another area of veterinary interest regarding toxicological testing is the evaluation of the safety of veterinary drugs for food animals, as well as the safety assessment of veterinary drug residues in human food of animal origin, in order to determine the doses that cause non-observed effect level (NOEL) and consequently to establish the acceptable daily intake (ADI) in human diet.

Toxicological testing laboratories now have to comply with strict official controls and inspections on animal use at any time that can be reinforced by relevant legislation. In most European countries, veterinarians play a pivotal role in toxicity testing and are required by law to be employed by designated establishments that undertake experiments on animals, including toxicological studies. More specifically, a named veterinary surgeon is responsible not only for the health and well-being of laboratory animals but also to advise on the selection of adequate *in vivo* models and ensure that priority is given to the use of alternative methods during the local ethical review process. Furthermore, the refinement of experimental techniques and husbandry approaches should be one of the priorities of a veterinary surgeon working in animal units, especially where regulated procedures take place, and distressed animals should be kept under very close supervision by a veterinarian. A similar approach is used in the U.S., where all institutes are also obliged to establish an Institutional Animal Care and Use Committee (IACUC), where the participation of a trained and experienced veterinary surgeon is essential to ensure that all aspects of the 3 “R”s (replacement, reduction and refinement) (Russell and Burch, 1959) have been followed before approving animal use for toxicological studies.

A veterinarian with specific professional or scientific interest in toxicology should be aware of the alternative methods in toxicity testing, advise on replacement and refinement of laboratory animals used in toxicological studies and safeguard animal welfare. The main aims of this chapter are to outline the major aspects of *in vivo*

and *in vitro* models in toxicity testing and to give a brief overview of endpoint determination. The principal focus will be to highlight some of the current *in vivo* and *in vitro* models available in toxicity testing and inform on new technologies and approaches used in this field.

## IN VIVO MODELS IN TOXICITY TESTING

### Introduction

With the continuous development of new chemicals and pharmaceutical products, laboratory animals have become important and well-established tools for the generation of *in vivo* toxicological data. Originally, *in vivo* experiments were aimed at the prediction of acute systemic toxicity usually in rodents. Currently, more sophisticated, targeted and multi-species approaches with well-defined endpoints and experimental protocols are applied to toxicological studies, especially for regulatory testing.

As the science of toxicology evolves, an increasing number of *in vitro* alternative tests have been validated or they are currently under development. However, in some cases, animal models in toxicity testing are irreplaceable, especially in the tests required by the regulatory authorities to protect human and animal health. Furthermore, there is public demand to know the toxicity risks posed in everyday life, which necessitates the use of animal models comparable to humans.

Although animals are relatively expensive experimental models compared to the alternative methods discussed later, there are several important reasons for their continued use. First, there is extensive information available on their normal biochemical and physiological properties. Second, the published data from the measurement of toxicological endpoints *in vivo*, using models of relevance to humans and domestic/farm animals, makes animal testing a valuable tool to predict toxicity.

The animals that are most commonly used in toxicological testing are rodents and rabbits (Table 5.1). Cats and dogs are used less frequently in toxicity testing (and mostly in preclinical toxicology or phase I pharmacological studies), whereas non-human primates are rarely used and mainly to study metabolism of toxic compounds. In this table are not included the studies conducted on companion animals to determine safety limits in products that are directly applied to cats or dogs (EPA, 1998a). The interested reader is referred to some more specialized books regarding animal toxicity testing (e.g., Arnold *et al.*, 1990; Gad, 2006) and some useful websites relevant to regulatory toxicological testing (e.g., <http://www.epa.gov/ocspp/pubs/>

[frs/publications/Test\\_Guidelines/series870.htm](frs/publications/Test_Guidelines/series870.htm) and <http://lysander.sourceoecd.org/vl=23534020/cl=14/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm>).

### Animal welfare in toxicity testing

Animal welfare legislation is currently applicable in most countries to prevent misuse of animals in toxicological testing. In the U.S., the Animal Welfare Act as amended (7 USC, 2131-2156) provides guidelines and protection to animals used for scientific purposes including toxicity testing. In Europe, Directives 86/609/EC and 2003/65/EC have been recently replaced by Directive 2010/63/EU. The latter is based on the principle of the 3Rs and aims to upgrade the minimum standards for laboratory animals by introducing appropriate animal welfare indicators and better training and information for animal handlers. Furthermore, it is necessary that there is explicit and easy-to-follow documentation in each project license involving toxicological testing in animals, where the steps followed in the case of animal distress are described. Testing new chemical compounds on *in vivo* models can lead sometimes to pain, discomfort or animal distress. Animals exhibiting signs of suffering should be examined by a veterinarian or trained personnel and, if there is a good scientific reason to continue the study, administration of analgesics should be considered. Other options available are the euthanasia of the distressed animals and the decrease or the discontinuity of the test compound dose.

In general, there is a trend for improvements to enhance animal welfare, not only in animal housing (e.g., breeding, handling and feeding) but also in the same regulatory toxicity testing guidelines, by applying humane endpoints and decreasing the number of animals required (Combes *et al.*, 2004).

Handling laboratory animals and administering toxicological compounds is a stressful procedure for both the animals and the designated personnel. In recent years, there has been an attempt to apply the highest ethical standards not only by introducing an excellent environment and a social and complex housing but also by reducing and improving any invasive techniques used on laboratory animals.

Implanted biosensors are now available that permit the continuous telemetric monitoring of physiological and biochemical parameters in experimental animals, and have been proved useful in toxicological studies to minimize not only the artifacts due to animal handling and restraint but also to improve animal welfare. In addition, non-invasive and currently expensive methods such as magnetic resonance imaging (MRI) and nuclear magnetic resonance spectroscopy are available for the

TABLE 5.1 Examples of animal models used in selective toxicity tests

Order	Species	Toxicity tests	References
Rodentia	Rat	Developmental toxicity	(EPA, 1998b, 2000; OECD, 2001b)
		Carcinogenicity	(OECD, 2009a,b)
		Cutaneous toxicity	(OECD, 1987)
		Genotoxicity	(OECD, 1984; EPA, 1998c)
		Immunotoxicity	(IPCS, 1996; EPA, 1998g)
	Mice	Neurotoxicity	(OECD, 1997c; EPA, 1998d, e)
		Developmental neurotoxicity	(EPA, 1998f; OECD, 2007)
		Reproductive toxicity	(EPA, 2000; OECD, 2001a)
		Carcinogenicity	(OECD, 2009a,b)
		Skin sensitization	(EPA, 2003)
		Genotoxicity	(OECD, 1984; EPA, 1998c)
		Immunotoxicity	(IPCS, 1996; EPA, 1998g)
	Hamsters	Neurotoxicity	(OECD, 1997c; EPA, 1998d, e)
		Reproductive toxicity	(OECD, 2001a)
	Guinea pigs	Carcinogenicity	(Gad, 1998)
		Genotoxicity	(Loomis and Hayes, 1996)
		Cutaneous toxicity/skin sensitization	(OECD, 1987; EPA, 2003)
	Rabbit	Developmental neurotoxicity	(Kaufmann, 2003)
		Developmental toxicity	(EPA, 1998b; Foote and Carney, 2000; OECD, 2001b)
Lagomorpha	Rabbit	Cutaneous toxicity	(OECD, 1987; Auletta, 2004)
		Reproductive toxicity	(Foote and Carney, 2000)
Avian	Hen	Neurotoxicity	(OECD, 1995a, b)
Swine	Minipigs	Cutaneous toxicity	(Auletta, 2004)
		Developmental neurotoxicity	(Kaufmann, 2003)
Canine	Dog	Carcinogenicity	(Loomis and Hayes, 1996)
		Cutaneous toxicity	(Vail <i>et al.</i> , 1998)
		Neurotoxicity	(EPA, 1998e)
		Developmental neurotoxicity	(Kaufmann, 2003)
		Reproductive toxicity	(FDA, 1982)
Non-human primates	Monkey	Developmental toxicity	(Buse <i>et al.</i> , 2003)
		Cutaneous toxicity	(deBlois and Horlick, 2001)

TABLE 5.2 Proposed administration routes of test compounds in laboratory animals based on the medium of exposure

Medium of human and domestic/farm animal exposure to toxicant	Administration route
Food commodities	Oral
	Oral
	Inhalation
Water	Dermal
	Inhalation
Air	Inhalation
Household/environmental surfaces	Oral
	Dermal

visualization of pathological findings and determination of the distribution of a test chemical in laboratory animals, respectively, which further contribute to improved animal welfare.

### Routes of test compound administration

Toxicity testing in animal models is most useful if it imitates the human or domestic/farm animal route of exposure to chemical agents. Based on the medium of exposure in human and domestic animals, it is possible

to decide which is the administration route of choice in animal toxicological tests (Table 5.2). Depending on the route of administration, experimental evaluation may differ because of variation in the absorption, metabolism and elimination of a compound. Oral exposure can lead to absorption by the digestive system and metabolism by the liver, whereas, following inhalation, a toxic compound is more likely to be absorbed by the respiratory system. Metabolism of xenobiotics can also occur in placenta, in the test dam and fetus. This can lead to changes in the balance of parent compound and metabolites, complicating the picture even more in case of developmental toxicity tests.

This metabolism of administered chemical should be relevant to human or domestic animals and is critical for risk assessment exercises. The final toxic effect will depend on a balance between the level of toxic agent reaching the target tissue and its rate of elimination and/or bioinactivation by mixed function oxidases, serum hydrolases or binding to serum proteins. Furthermore, there are interspecies differences regarding the metabolism of xenobiotics (Nebbia, 2001). For example, cats are at high risk of developing hepatotoxicity especially after paracetamol administration. This is due to differences in



bioactivation of paracetamol, which occurs only in cats through *N*-hydroxylation with the help of cytochrome P450 2E1, during the oxidative reactions in phase I transformation (Nebbia, 2001). Ruminants are less susceptible to organophosphates (OPs) such as parathion than monogastrics, because the rumen microflora plays an important role by reducing the nitro group of OPs to an amino group (Nebbia, 2001).

The majority of toxicological studies commonly employ administration of the agent in animal feed or water or by stomach intubation (i.e., by gavage) in order to imitate a known or potential human or domestic animal exposure. The use of oral gavage is commonly used in administration of high doses of xenobiotics and in developmental toxicity tests, but is less practical in the case of long-duration studies. Inhalation is used when there is a need to duplicate industrial or environmental exposure to dusts, aerosols and fumes. In this case, nose, head or whole body exposure chambers are used, depending on the exposure time. For cutaneous administration, a toxic agent may be injected intradermally or simply applied topically on the skin or ears and sometimes covered with bandages. In case of experimental studies where the need for complete absorption of a tested compound is considered essential, parenteral routes of administration (intraperitoneal, intramuscular, intravenous and subcutaneous) are selected. However, the solubility and bioavailability of the tested agent can also influence the degree of absorption and how much of it is directly available in a laboratory animal.

Toxicokinetic and pharmacokinetic information on tested compounds and their comparison among laboratory animals and humans are also important to determine dosing parameters and improve the toxicological data obtained. The administered dose in toxicological studies should be decided taking into account many physicochemical parameters of chemical agents, biological differences between species, previously published data and after careful planning or preliminary experimentation.

## Reproductive toxicity tests

Reproductive toxicity testing is based on the measurement of reproductive functional and structural defects caused by toxic agents in both males and females. The toxicity endpoints most frequently studied in reproductive *in vivo* testing are summarized in Table 5.3. Rats and, to a lesser extent, mice are the species of choice in reproductive toxicological testing because they are considered inexpensive compared to bigger mammals (EPA, 2000; OECD, 2001a). On the other hand, small rodents need to be euthanized even for simple endpoints such as collection of sperm, whereas rabbits can be sampled

TABLE 5.3 Selective endpoints applied to laboratory animals during reproductive toxicity testing

Female	Male
Reproductive tract morphology	Sperm structure/morphology
Reproductive tract receptors	Sperm motility/viability/count
Ovum properties	Sperm DNA integrity
Recovery of blastocysts	Hormonal balance/Receptor interactions
Hormonal balance/receptor	Fertility testing interactions
Length and normality of estrus cycle	
Fertility testing	
Uterine condition	
Implantation	
Lactation	
Maternal behavior	

regularly without being sacrificed, making them an alternative in reproductive toxicological tests (Foote and Carney, 2000). Some of the disadvantages of using rabbits are the higher cost due to greater amounts of chemical compounds administered and the increased cases of abortions because of the relatively high incidence of gastrointestinal dysfunction. For example, rabbits are poor models for veterinary residue testing and, more specifically, for antibiotics because these compounds have been found to cause diarrhea and consequently abortion (Barlow *et al.*, 2002).

Dogs have been occasionally used for reproductive toxicity testing, because the physiology of their reproductive system in both sexes has been extensively studied, contributing to significant background knowledge (FDA, 1982). However, the dog is not the model of choice in reproductive toxicity testing because it is a rather expensive animal model and the number of litters is not as high as in rodents. The main difficulty in running reproductive toxicological tests is the requirement for an estrous-synchronized population. Both the induction of ovulation by hormonal treatment and artificial insemination mainly in rabbits and dogs can overcome this problem and allow advanced planning for animal fertility studies.

Since exposure to chemicals can occur throughout life, a multi-generation study which extends over at least two generations, using a single type of laboratory rodent, is desirable in order to reveal reproductive toxicological data (OECD, 2001a; Barlow *et al.*, 2002; Garg *et al.*, 2011; Estevan *et al.*, 2011), although this approach requires large numbers of animals and it is time consuming.

## Developmental toxicity testing

Developmental toxicity testing is primarily used to determine hazard regarding the potential effects of

prenatal exposure on the developing fetus. These studies focus on functional and structural changes that can be observed throughout the development from zygote to neonate. The most important developmental phase is the organogenesis period that is always taken into account in developmental toxicity testing. Based on these studies, chemical compounds can be categorized as teratogenic and/or fetotoxic by recording structural malformations, developmental retardation and/or mortality, respectively. The vast majority of teratogenic chemical agents have been identified using rodent experimental models (EPA, 1998b, 2000; OECD, 2001b). However, the failure of rodents to detect teratogenic signal on some occasions and the similarities in placentation and pregnancy physiology between humans and rabbits led to the use of the rabbit as a second model for assessing the effects of toxic compounds on development (EPA, 1998b; Foote and Carney, 2000; OECD, 2001b). Furthermore, although non-human primates have been suggested as models for teratological testing (Buse *et al.*, 2003; Faqi, 2011), they have several limitations such as a long gestation period, only single or twin offspring, high rates of abortion and ethical constraints.

The detection rates for veterinary pharmaceutical agents demonstrated to be teratogenic/fetotoxic were found to be 55–79% using individual species (Hurtt *et al.*, 2003). However, when the rat and rabbit data were both considered, there was a significant increase in detection rate to almost 100%, suggesting that in the absence of teratogenicity in rat, a second species developmental test in lagomorpha is required to provide high standards of public protection (Hurtt *et al.*, 2003). The interested reader can retrieve some more information on reproductive and developmental toxicity testing by referring to a specialized book (Gupta, 2011).

### Cutaneous toxicity testing

The aim of *in vivo* assays for cutaneous toxicity is not only to assess potential acute local irritation but also to evaluate acute, subchronic and chronic systemic toxic effects. During cutaneous toxicity tests, animals are monitored for skin reactions/dermal effects, clinical, gross or microscopic pathological findings depending on the duration of a toxic compound administration and the observation period.

The albino rabbit has been until recently the animal model of choice because of the high permeability and sensitivity to toxic agents exhibited by its skin, which sometimes led to overprediction showing little relevance to human irritation (Auletta, 2004). Currently, albino rats and occasionally guinea pigs are considered preferable species to assess local irritation. Traditionally, guinea pigs and mice have been used also to perform

sensitization tests (EPA, 2003). On the other hand, long-term cutaneous toxicity studies usually require the use of a rodent (albino rat and mouse) as well as a non-rodent model. Minipigs have been proved to be a reliable non-rodent species because their skin demonstrates many physiological similarities with those of humans and pigs (Auletta, 2004). Other non-rodent models are dog and non-human primates, commonly used to test the metabolism of toxic agents and the safety of newly designed recombinant pharmaceutical products (Vail *et al.*, 1998; deBlois and Horlick, 2001).

### Genotoxicity testing

The aim of genotoxicity testing is to detect gene damage induced by the test compound, by measuring chromosome aberration and breakage, point mutation, and other DNA and chromosomal effects *in vivo*. The host-mediated assay is based on the inoculation of a microorganism into a rodent such as mouse, rat or hamster and subsequent assessment of the point mutations found in the microorganisms, after certain treatment of the rodents with the potential mutagen (Gabridge and Legator, 1969; Dhillon *et al.*, 1995; Loomis and Hayes, 1996). On the other hand, to identify chromosome breakage, male rodents are treated with the test compounds and, after mating with untreated females, fetal mortality and survival are recorded (OECD, 1984; EPA, 1998c).

The mouse spot test is capable of detecting somatic gene mutations and is based on *in utero* exposure to the tested chemical. This *in vivo* mutation test works by monitoring the appearance of colored spots in the coat of the animal that may appear due to altered or lost specific wild-type allele in a pigment precursor cell (EPA, 1998h).

A popular *in vivo* test to identify genetic risks is the rodent bone marrow micronucleus test, although it is not the most sensitive test and it does not improve predictivity of rodent genotoxicity when combined with *in vitro* tests (OECD, 1997a; Zeiger, 1998). However, this *in vivo* test is widely used because it is relatively easy to perform. Another *in vivo* test that has been validated and recommended by the Organisation for Economic Cooperation and Development (OECD, 1997b) is the rat liver unscheduled DNA synthesis (UDS) test. It is worth mentioning that none of the above approaches are suggested to be used individually to predict genotoxicity. Negative or positive results should always be confirmed first by *in vitro* or non-mammalian mutagenicity tests.

### Carcinogenicity tests

*In vivo* assays of carcinogenicity examine the possibility that a tested agent might cause tumors and other

chemically related effects in one or more animal species. Currently available is the rodent carcinogenicity test (OECD, 2009a). This test runs for up to 2 years and involves the use of three different concentrations, one administration route and both sexes. The results are based on clinical chemistry, gross and histopathological analysis of more than 40 tissues and organs in order to determine the site, the number and type of tumors (OECD, 2009a, b).

One serious disadvantage of using mice in carcinogenicity testing is their tendency to present high incidence of spontaneous liver or lung tumors in some strains, leading occasionally to inconclusive results (Gad, 1998). Furthermore, because of the long duration of carcinogenicity tests and therefore the old age of laboratory animals, it means that the natural occurrence of tumors increases, making it difficult to distinguish between a real treatment and a background carcinogenic effect. In addition, the most common mechanisms of tumor development in rodents may not be relevant to human carcinogenicity, posing limitations to any attempt to use *in vivo* data for carcinogen risk assessment.

Tumorigenic tests using dogs are limited because of the high cost and the long duration of the studies (up to 7 years). Furthermore, dogs demonstrate high susceptibility to aromatic amines and therefore care should be taken when using these animals for the evaluation of potential carcinogenic compounds of this category (Loomis and Hayes, 1996).

## Neurotoxicity

The neurotoxic potential of chemical compounds can be assessed by determining relevant effects on the autonomic or central nervous system (CNS), not only in adult but also in developing animals. Clinical signs including changes in behavior (e.g., movement, motor coordination or reflexes, paralysis, tremor, learning and memory), neurochemical (e.g., activity of enzymes associated with neuropathies, cell signaling pathways, synthesis, release and uptake of neurotransmitters), neurophysiological (e.g., electroencephalography, nerve conduction velocity) and neuroanatomical effects are commonly explored.

Rodents are the most frequently used animal models and there are a considerable number of available guidelines to examine the neurotoxicity of xenobiotics on them (OECD, 1997c; EPA, 1998d, e). However, an important issue related to the selection of animal model in neurotoxicity testing is the delayed onset of effects manifested by toxic agents. For example, in the case of assessment of the potential of OPs to produce delayed neuropathy induced by organophosphate (OPIDN), as rodents are not the most sensitive models, the use of the hen model is recommended (OECD, 1995a, b). The two protocols

used in the hen model involve either acute or repeated dosing for up to 28 days and determination of the enzymes acetylcholinesterase (AChE) and neuropathy target esterase (NTE), clinical observation and histopathology of the CNS (OECD, 1995a, b).

Furthermore, the need to address the specific risks of the developing nervous system has led to the application of more specific developmental neurotoxicity testing guidelines in rats (EPA, 1998f; OECD, 2007). Although rodents are the animal model of choice, the main problem using them in developmental studies is that the period of enhanced brain growth takes place during the first 10 days after birth, whereas in humans, dogs, guinea and mini pigs, this period is completed prenatally. In conclusion, for predicting developmental neurotoxicity risk in humans, the rat is the recognized model. However, dogs, guinea and mini pigs are also widely used to test pharmaceutical compounds that target children, due to the limitations of the rodent model (Kaufmann, 2003).

## Immunotoxicity

Immunotoxicity tests are designed to detect adverse effects of xenobiotics on the immune system including all the relevant cells, organs and mechanisms of immune response, whether or not there is a measurable disturbance in host resistance. Toxic substances can directly or indirectly cause immunotoxicity, either by inhibiting the enzymatic activity of esterases and serine hydrolases in the immune system or by chronic alteration in metabolism of organs in the immune and nervous system, respectively. Some of the approaches, tests and endpoints used to assess *in vivo* immunotoxicity are presented in Table 5.4 (EPA, 1998g; Barlow *et al.*, 2002; Galloway and Handy, 2003; OECD, 2008).

Laboratory rodents, especially mice, are used in toxicological tests because basic immunological studies were mainly conducted in this species. The immune elements and interactions in rodents and humans are very similar and if the toxicokinetic properties of tested chemicals are close, then the use of mice or rats is recommended (IPCS, 1996; EPA, 1998g).

Chlorpyrifos, an OP with distinctive toxic effects on neurodevelopment, was reported to affect intracellular signaling cascades involved in differentiation and proliferation of T lymphocytes (Blakely *et al.*, 1999) and has recently been implicated as an endocrine disrupting chemical, thus possibly affecting endocrine-mediated immunity. In most experimental studies, cholinergic doses of OPs were shown to reduce immune activity whereas non-cholinergic ones resulted in an increased response, although there is still research to be done to this direction (Pruett, 1992; Galloway and Handy, 2003; Sharma, 2006).

TABLE 5.4 Immunotoxicity endpoints, methods and approaches used for *in vivo* toxicity testing

Tests	Methods	Endpoints
Non-functional tests of immunotoxic response	Immunopathology	Lymphoid organ weight
	Routine hematology	Histopathology of lymphoid tissues, including bone marrow Immunocyte viability/differential count Immunoglobulin levels
Functional tests of immunotoxic response	Measurement of humoral immunity	Antibody response
	Measurement of non-specific immunity	Antibody plaque forming Neutrophil and monocyte numbers
	Measurement of cell mediated immunity	Phagocytic activity of macrophages Mitogen-induced cell proliferation
	Host resistance assays	Natural killer cell activity Mixed lymphocyte reaction Delayed hypersensitivity
		Mortality and organ histopathology due to bacterial, virus and parasitic infection

### Transgenic animals in toxicity testing

The recent advances in genetic engineering techniques led to the development of a number of transgenic *in vivo* models that can be used in toxicity testing, mainly in carcinogenicity and mutagenicity, as well as for the study of xenobiotic metabolism. These *in vivo* systems permit the study of toxicological effects of tested compounds on foreign genes of human or other origin that have been genetically transferred to produce transgenic animals, by transgenesis or targeted gene modification. From the point of view of toxicity testing, it is considered that such models will lead faster to results that are more representative of human response to xenobiotics. In addition, transgenic mice can bring significant animal welfare benefits, because they are able to reduce the group size needed in experiments and replace testing to other species, including non-human primates. Finally, these models are also excellent experimental systems and able to address and answer specific mechanistic questions in toxicology more efficiently (Valancius-Mangel and Doetschman, 1999). However, the use of "humanized" animal models in toxicological studies is an expensive process because it involves higher costs for their development and breeding. There is also concern that the human genome, once transferred to laboratory animals, may express the same proteins, but it does not guarantee that the protein will have similar function with that found in humans. In addition, there is limited background information on transgenic animals, making the interpretation of data in toxicological studies difficult (Valancius-Mangel and Doetschman, 1999).

The main transgenic genotoxicity systems used in toxicology to follow up *in vitro* genotoxicity positives are the Muta Mouse and Big Blue rat/mouse models,

in which the genome is "tagged" with the markers lacZ ( $\beta$ -galactosidase) and lacI, respectively (Gossen *et al.*, 1994; Winegar *et al.*, 1994; Wahnschaffe *et al.*, 2005a, b). Aflatoxin B1, a mycotoxin commonly found in human food and animal feed, has been tested in transgenic C57BL/6N mice and caused lacI mutation in animals' kidney and liver, proving the genotoxicity effect of the mycotoxin and demonstrating the usefulness of transgenic mice in toxicology because not only can this approach quantify mutant frequencies, it also identifies specific types of mutational events (Autrup *et al.*, 1996).

Several models are available to assess the carcinogenicity of a test compound: these include (1) inactivated tumor suppressor gene and DNA repair gene models such as p53<sup>+/−</sup> and XPA<sup>−/−</sup> knockout mice, and (2) oncogene activation models like the TgAC and the ras H2 transgenic mice (Valancius-Mangel and Doetschman, 1999).

Other important transgenic systems with toxicological application that have been designed to express/lack one or more of the proteins involved in metabolism of xenobiotics and their use has led to a better understanding of metabolic activation and transformation. Most of these transgenic models express human cytochrome P450 (CYP1A2, CYP1B1) or are knockout mice (CYP2E1/CYP1A2 null mice) (Valancius-Mangel and Doetschman, 1999).

### Limitations and implications of animal use in toxicological testing

The closer a test is to the phylogenetic species of concern, the more relevant the information obtained and the higher its predictivity. There are basic similarities



in biology and xenobiotic metabolism between humans and the different mammalian species used in toxicity testing, making *in vivo* testing the most reliable approach in chemical safety assessment (Knudsen, 1999). However, because of species and strain variability in uptake, metabolism and elimination of xenobiotics, no single type of animal is an ideal model for predicting human or domestic animal toxicity. Consequently, multiple *in vivo* models are usually required to determine toxicity of agents and to contribute to more precise risk assessment, which may have ethical implications.

Currently, there is a trend in using well-established inbred (immortal clones of genetically identical individuals) strains of rodents, in order to increase the sensitivity of animal experimentation in toxicological testing by maximizing the reproducibility of the results. While this approach can reduce animal numbers, the results may not be representative of a heterogeneous population; using a heterologous group of one species with low inbreeding may therefore be a more realistic approach reflecting the impact of a toxic compound on a broader spectrum of genes.

In the last three decades there has been an increased sensitivity to ethical issues involved in use of animals for toxicity testing. The tactics of mass demonstrations by animal protection and animal extremist groups has created an atmosphere where many institutions, organizations or companies and their employees are reluctant to support or carry out any animal testing, before making sure that an alternative test does not exist. In addition, there are economic arguments against the use of animals in toxicity testing. Approaches using animals are undoubtedly more time consuming and more costly to run, compared to *in vitro* models. There are certain standards that should be maintained from laboratories undertaking toxicity testing experiments, such as Good Laboratory Practices (GLP), not only from an animal welfare prospective but also from an experimental reproducibility approach, something that adds to the cost.

Furthermore, the quantity and quality of animal feed should also be considered because several studies demonstrated that the nutritional status can interfere with the reproducibility of the results obtained from animal models used in toxicity testing (Kacew, 2001). Other factors contributing to data quality are the health status of the animal stock (specific pathogen free, germfree or with ordinary microflora) and the laboratory experience in handling and caring for the stock. In conclusion, toxicological animal testing, although a valuable tool to predict human and domestic/farm animal toxicity of chemicals, is labor intensive, time and space consuming and raises important animal welfare concerns and uncertainties. For these reasons, there has been increasing interest in the development of *in vitro* alternatives that can help to reduce the use of animals in toxicity testing.

## IN VITRO MODELS OF TOXICITY TESTING

### Introduction

*In vitro* cellular models are relatively inexpensive and easy to maintain and manipulate compared to animal models. *In vitro* methods allow the study of direct cellular effects of toxins on specific cell or tissue types in a controlled environment.

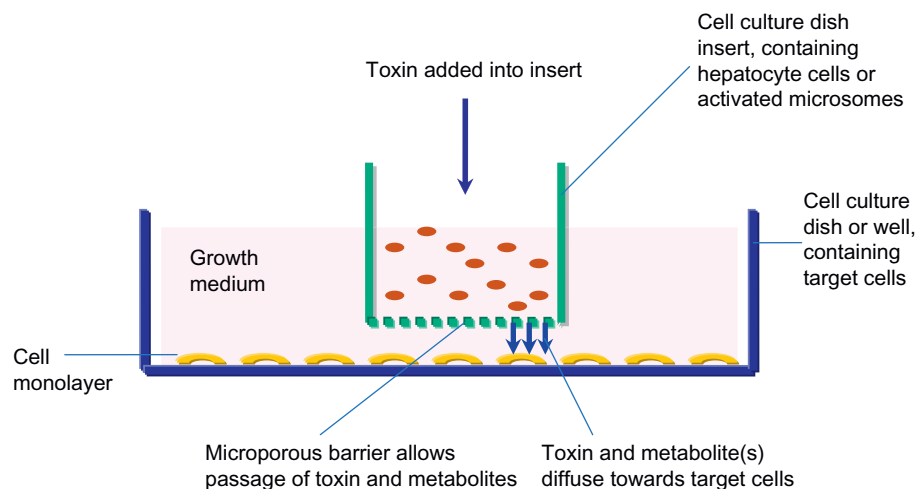
However, the main disadvantage of *in vitro* systems over animal models is the lack of systemic effects such as an appropriate balance and supply of growth factors and a system of xenobiotic metabolism and elimination of toxins. The former can be at least partially addressed by adding appropriate growth factors and the latter can be addressed by the use of metabolic activation systems such as the introduction of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-activated microsomes or a hepatic cell line in a cell culture well insert fitted with a filter (Figure 5.1). The toxin is introduced into the insert and a mixture of metabolized and non-metabolized toxin (but not the microsomes or the hepatic cells) diffuses through the filter into the growth medium containing the target cells. Another disadvantage is that many cellular systems lack the complexity of cell-cell interactions in tissue, although this can be addressed to varying degrees using co-culture systems or organ culture, which are discussed later.

### Types of cell culture system used in toxicity testing

Before discussing the assays used in the *in vitro* systems it would be useful to discuss the principles, strengths and weaknesses of the main types of *in vitro* system. All cell cultures need to be prepared and maintained under sterile conditions in order to reduce microbial contamination. Cells are maintained under defined conditions of humidity, pH and temperature. They are grown in a specific growth medium, which may have a number of supplements, such as antibiotics, glutamine, serum, etc., which are different for each cell line. Growth conditions should be optimized prior to experimental work. Excellent reviews on practical aspects of cell culture can be found in the following sources (Cohen and Wilkin, 1996; Shaw, 1996; Masters, 2000; Davis, 2002; Gardner *et al.*, 2004).

There are a number of types of cell culture available for *in vitro* testing that offer various degrees of complexity and relatedness to the *in vivo* situation. In order of increasing complexity and genetic similarity to the tissue of origin, these include permanent cell lines, primary cultures and organotypic cultures (Figure 5.2) (for





**FIGURE 5.1** Metabolic activation in cell culture systems.

Increasing complexity and similarity to <i>in vivo</i> systems	Culture type	Suitability/limitations
	Mitotic cell lines	Medium to high throughput studies of basal toxicity (e.g., membrane damage, viability, etc.) and cell proliferation. If immortalized, many cell lines are tumor-like. Limited cell–cell interactions and drug metabolism.
	Differentiating cell lines	Medium to high throughput screening and mechanistic studies of developmental toxicity and target cell specific toxicity. Often short-lived. Limited cell–cell interactions and drug metabolism.
	Primary cell cultures	Developmental or target cell-specific toxicity. Genetically more similar to target system but generally heterogeneous and short-lived. Can be used as co-culture systems (e.g., reaggregates) to simulate cell–cell interactions of target tissue but usually have limited drug metabolism.
	Organotypic/whole organ cultures	These are tissue slices or cultures organs that can maintain cell interactions and tissue function. Generally unsuitable for medium to high throughput analysis and may exhibit limited drug metabolism.

**FIGURE 5.2** Organization of a tiered system for *in vitro* toxicity testing.

reviews see [Noraberg, 2004](#); [Spielmann, 2005](#); [Sundstrom et al., 2005](#)).

Permanent cell lines are mitotic and can be finite, established or clonal in nature. They have the advantage that they are relatively easy and inexpensive to maintain compared to animals and they are amenable to cryopreservation under liquid nitrogen. However, if maintained through high numbers of divisions, there is

an increasing likelihood of genetic drift that might affect phenotypic properties of relevance to toxicity testing.

Finite cell lines are normally derived from primary cultures (see below) and can survive for 40–50 divisions before finally dying (e.g., fibroblasts). Established cell lines are effectively immortal, having been transformed with a virus, a mutagen or spontaneously. These are generally tumor like in nature; some widely used examples

include mouse 3T3 fibroblasts, HeLa cells and Chinese hamster ovary (CHO) cells. However, several cell lines can be induced to differentiate, making them potentially useful models of specific stages of development, for example the use of nerve growth factor or retinoic acid to induce a neuronal phenotype in cultures of rat PC12 pheochromocytoma and human SH-SY5Y neuroblastoma cells, respectively (Fujita *et al.*, 1989; Presgraves *et al.*, 2004).

Clonal cell lines are derived from the mitotic division of a single cell seeded in a sterile microtiter plate by limiting dilution as used in the cloning of hybridoma cell lines. Thus, cell lines can be cloned to exhibit a specific trait (e.g., high levels of specific receptors, drug resistance, etc.). While a homogeneous response to toxin treatment might then be expected from such a cell line, there is the risk of losing other features of a more heterogeneous population. In some cases, clones of transfected cell lines provide useful tools for mechanistic studies of cell differentiation and/or toxicity. For example, some cell cultures have been transfected with the certain cytochrome P450 transgenes to make them metabolically more competent during long-term studies (Tzanakakis *et al.*, 2002).

Primary cultures are derived by a combination of mechanical and enzymic disruption of the tissue of interest, which releases a collection of cells that resembles the tissue of origin closely both genetically and in terms of cell heterogeneity. It is then possible to enrich in specific cell types by using either selective culture media and/or cell growth inhibitors, immunomagnetic beads or fluorescence-activated cell sorting (FACS). If a monoculture is desirable (i.e., predominantly one specific cell type), the resultant culture would then need to be screened for cell-specific markers to determine purity/enrichment of the preparation, as a pure culture is rarely achieved. Typically, this would take the form of monitoring cultures for the expression of a unique morphological or molecular trait of the desired cell type to determine the level of enrichment. For example, expression of glial fibrillary protein (GFAP) and aster-like morphology would be good markers for astrocytic glial cells. Cells may be cultured as monolayers or, if a system simulating cell-cell interactions of the tissue of origin is required, cells may be cultured in suspension with mild agitation or using rolling cell culture. Under these circumstances, cells form clusters, spheroids or reaggregates that may continue to grow/proliferate over a period of several days or weeks, making them amenable to studies of long-term effects. For example, brain reaggregates prepared in this way have been maintained for up to several weeks and used in studies of pesticide toxicity (Sales *et al.*, 2000). However, many primary cultures tend to be very short lived. However, primary cell cultures do not necessarily exhibit identical cell-cell interaction patterns

as those in the tissue of origin and cell types that divide more rapidly could become more predominant than they would be *in vivo*.

A more recent development has been the use of stem cell lines as models for developmental toxicity testing (Hansen and Inselman, 2011). These cell lines are normally maintained in growth media containing mitogens, and can be induced to differentiate into different cell types (e.g., cardiomyocytes, pancreatic and neural cells) by removal of mitogens and/or the addition of specific trophic factors (Rolletschek *et al.*, 2004). Embryonic stem cells (ESCs) have the potential to differentiate into any cell type whereas progenitor cells are already committed to follow a specific developmental pattern. Alternatively, progenitor cell lines can be established; for example, neural progenitor cells can be induced to differentiate into a co-culture of neuronal and glial cell types, simulating the early stages and cell-cell interactions of neural development *in vivo* (Breier *et al.*, 2010). However, while stem cell cultures may represent excellent *in vitro* models for studying developmental toxicity, they are unlikely to exhibit identical cell-cell interactions and growth patterns as those in mature adult tissue.

One way to address this issue may be to develop a post-mitotic system containing pre-differentiated cells prior to the addition of toxin. However, this issue may be better addressed by using organotypic cultures. In this case, tissue slices (typically 200  $\mu$ m thickness) are cut from fresh tissue on a microtome, then subsequently rinsed and cultured in growth medium with agitation, as discussed above. Such slices maintain the complexity of cell-cell interactions and extracellular matrix composition of the original tissue and in some cases can survive up to several weeks (Sundstrom *et al.*, 2005). Nevertheless, even this kind of cellular system lacks the systemic interaction with the immune and circulatory systems that would occur *in vivo* and is not, therefore, a complete substitute for *in vivo* testing. However, using cell cultures as part of a tiered system of increasing complexity from *in vitro* to *in vivo* measurements would improve throughput, decrease costs and allow drastic reduction in the use of live animals in screening compounds for potential toxic effects. Some examples of *in vitro* toxicity testing systems in current use are indicated in Table 5.5.

### Endpoint determination for *in vitro* testing systems

A good *in vitro* testing system should be sensitive but at the same time yield low levels of false-positive and false-negative results. It should have endpoint measurements: that (1) show dose-response relationships for a given toxin, (2) reflect and are predictive of the *in vivo* pattern of toxicity for a given group of agents, (3) are objective

TABLE 5.5 Examples of cell culture systems used to model specific types of toxicity

Model	Description and comments
Neurotoxicity	Differentiating neural cell lines (e.g., human SH-SY5Y and rat PC12 neuroblastoma). Primary cultures, whole rat brain reagggregates and organotypic brain slice cultures (Sales <i>et al.</i> , 2000).
Hepatic toxicity	Human hepatoma HepG2 cell line and subclones expressing CYP1A1, cell lines engineered to express single human or animal P450, primary hepatocyte cultures, longer-term collagen sandwich cultures, liver slices and isolated perfused liver (Worth and Balls, 2002).
Developmental toxicity	Whole rat embryo cultures, rat limb bud reagggregates cultures and mouse embryonic stem cell lines (Liebsch and Spielmann, 2002). Standard operating procedures (SOPs) available on the ECVAM-INVITTOX databases.
Dermal toxicity	Keratinocyte and fibroblast cell lines. Excised rat skin models and human EPISKIN™ and EPIDERM™ skin models (Fentem <i>et al.</i> , 2001).
Immunotoxicity	Antibody production and activation/proliferation of lymphocytes (Karol, 1998).
Genotoxicity	Mammalian cell gene mutation and chromosome aberration tests (EPA, 1998i, j).

and reproducible, and (4) have internal controls. It is also useful if the testing system involves rapid assays of toxicity, allowing medium to high throughput analysis and simultaneous testing of multiple compounds and/or doses. The testing system should also be relatively inexpensive and involve technology and skills that are easily transferable to other laboratory personnel. Importantly, for a testing system to be deemed reliable for the prediction of acute *in vivo* human systemic toxicity it should have been validated through a rigorous international multi-center validation program. For example, during the period 1989–1996, 97 international laboratories tested the same reference chemicals (which represent different classes of chemicals with varied human toxicity) in their own *in vitro* systems. The outcomes of these studies are available on an Internet database (URL <http://www.cctoxconsulting.a.se/meic.htm>) and various aspects of the overall study have been published in eight articles in *Alternatives to Laboratory Animals*. Over the past 15 years or so, recommendations governing validation of *in vitro* alternatives, including systems for measuring chronic effects, have been published and are regularly reviewed by international organizations such as ECVAM (European Centre for Validation of Alternative Methods), ICCVAM (the Interagency Coordinating Committee on the Validation of Alternative Methods) and OECD. The interested reader is referred to the following articles for more recent publications on validated systems for toxicity testing (Liebsch and Spielmann, 2002; Bhogal *et al.*, 2005).

The best way to achieve something approaching an ideal testing system is to include a battery of endpoint measurements in order to minimize the occurrence of false-negative and false-positive results. Endpoint determination should give an objective assessment of a cytostatic, cytotoxic or other functional effect. It should also be quantitative or reproducibly qualitative. The selection of endpoints chosen should enable the categorization of toxins in terms of their toxicity relative to other agents of the same or other groups, giving reproducible results in different laboratory settings.

Endpoints can take a variety of forms, including measurements of cell viability, metabolic activity, morphology, changes in protein and gene expression and/or altered subcellular distribution of markers of interest. Some of the main endpoints are summarized below and the reader is referred to Masters (2000), the INVITTOX (<http://www.invittox.com/>) and ECVAM (<http://ecvam-dbalm.jrc.ec.europa.eu/>) websites for further information and technical details about specific protocols and validated models used in toxicity testing *in vitro*.

### Cell viability

The traditional method of determining cell viability is to determine the cell density (cells/ml) in a hemocytometer chamber. While this is a very useful method for determining cell number for seeding in cell culture experiments, it does not distinguish between viable and non-viable cells following exposure to toxin. However, the proportion of non-viable cells can be determined in parallel by assessing the percentage of counted cells that take up the dye Trypan blue, which is excluded from viable cells. Though effective, this approach is not suited to high throughput, as measurement can be time consuming and is therefore best suited to small numbers of samples or treatments.

An alternative approach is to use dye uptake assays. For example, neutral red accumulates on the lysosomes of viable cells, after which it can be extracted from cells with an organic solvent and determined spectrophotometrically. A variation on this is to prelabel cells with the dye prior to treatment with toxin; reduced levels of absorbance compared to untreated control cells would be indicative of dye release due to membrane damage.

Appropriately equipped laboratories might choose to use a dual fluorescence assay to determine the proportion of live and dead cells. In one such system, fluorescein diacetate is taken up by viable cells and cleaved by intracellular esterases to release fluorescein producing green fluorescence in the cytoplasm, whereas propidium iodide only crosses the membrane of damaged cells and

stains the nucleus (DNA) producing red fluorescence (Coder, 1997). Specimens are observed using a fluorescence microscope and quantification can be achieved by image analysis or by FACS analysis.

### *Membrane leakage*

Leakage of macromolecule through the plasma membrane into the culture medium is an effective means of detecting early or late stages of membrane damage. Cells can be incubated in the presence of  $^3\text{H}$ -thymidine, which is incorporated into DNA during cell proliferation. Subsequent loss of  $^3\text{H}$ -labeled DNA would be indicative of cell lysis. An alternative approach is to label cells in the presence of  $^{51}\text{Cr}$  which binds to many cellular proteins. Loss of labeled proteins from treated cells would be indicative of membrane leakage/cell lysis. In this sense the  $^{51}\text{Cr}$  release assay is more sensitive than that for  $^3\text{H}$ -labeled DNA as the former will detect signs of damage to the membrane at a much earlier stage than the latter.

An attractive alternative to radioisotopic methods is the release of lactate dehydrogenase (LDH) into cell culture medium, which is measured spectrophotometrically. The assay shows sensitivity comparable to the  $^{51}\text{Cr}$  release assay and is amenable to microtiter plate format, which would facilitate medium to high throughput analysis.

### *Cell growth and proliferation*

Cell growth or proliferation can be measured in a number of ways.

#### *Cloning efficiency*

In the absence of sophisticated equipment, perhaps the simplest method is that of cloning efficiency. In this case, cells would be plated at a density of 100–200 per culture vessel and assessed microscopically for their ability to produce viable colonies after a given period of incubation in the presence and absence of toxin. This is one of the most sensitive methods used for testing the effects of toxins on the growth of mitotic cells.

#### *Mitogenicity*

This can be determined in a number of ways. For example, the mitotic index of cell cultures can be determined by counting the percentage of mitotic figures following staining of DNA with hematoxylin. Alternatively, the proportion of cells in S-phase can be determined by immunohistochemical staining of cells incubated with the thymidine analog 5-bromo, 2-deoxyuridine.

#### *DNA synthesis*

Another approach is the measurement of cell growth in toxin-exposed cells via the incorporation of

$^3\text{H}$ -thymidine into DNA (Flaskos *et al.*, 1994). This method gives a good quantitative assessment of the effects of toxins on cell proliferation, as the radioisotope is incorporated into cells during S-phase of the cell cycle. High throughput analysis requires the use of a cell harvester to lyse cells and transfer radiolabeled DNA onto filters prior to detection by scintillation counting. Alternatively, cell proliferation can be measured by the incorporation of 5-bromo, 2-deoxyuridine incorporated into S-phase cultured cells, and subsequent quantification by enzyme-linked immunoabsorbent assay (ELISA) (Lanier *et al.*, 1989). Total DNA content can also be determined by detecting fluorescence after incubation of cells with DNA binding dyes such as Hoechst 33258, using a spectrofluorimetric microplate reader or by FACS analysis (Downs and Wilfinger, 1983).

#### *Protein synthesis*

Cell growth can also be measured by total protein content or by protein synthesis. Protein content can be estimated by a number of dye binding assays in microtiter plate format, such as the bicinchoninic acid (BCA) assay (Tuszynski and Murphy, 1990). More rapid analysis can be achieved by fixing and staining cells directly with Coomassie brilliant blue. After washing, the dye is solubilized and the absorbance of protein bound dye measured spectrophotometrically. As a measure of total protein synthesis, cells can be incubated in the presence of radiolabeled amino acids such as  $^{35}\text{S}$ -methionine or  $^{14}\text{C}$ -leucine and TCA precipitable protein assessed for radioactive content by scintillation counting. If an assessment of specific protein changes is required, protein extracts can be separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and proteins stained with Coomassie blue or another protein staining dye. Changes in radiolabeled protein synthesis, which could be masked by the pre-existing protein pool, can be detected by autoradiography following exposure of dried gels onto X-ray film.

#### *Cell morphology*

Changes in cell morphology can be measured microscopically and are very useful in studies of mechanisms of toxicity. However, medium to high throughput analysis of toxicity would require the use of image analysis software to produce more consistent data. Specialist techniques such as Allen video-enhanced contrast differential interference contrast (AVEC-DIC) microscopy facilitate analysis of effects on living cells. Furthermore, high throughput assays could be developed that measure the underlying molecular changes determined from follow-up studies.

Morphological changes can take various forms, as indicated below.



### *Cell volume*

An increase or decrease in cell volume could indicate osmotic changes or may represent the early stages of cell death by necrosis or apoptosis. Cell death or viability changes would then need to be made to confirm the type of cell death occurring, as indicated earlier.

### *Cell shape*

Changes in shape may occur following exposure to a toxin. These could include rounding up, flattening, spreading or process outgrowth in cell culture monolayers. Such changes would give an initial indication of altered cell attachment, migration, proliferation or differentiation, indicating potential targets for follow-up molecular studies. For example, OP-induced changes in axon outgrowth in cultured neurons indicated possible changes in proteins associated with axon growth and maintenance, which were then targeted in molecular studies (Hargreaves *et al.*, 2006).

### *Membrane integrity*

Changes in membrane integrity may be indicated by surface blebbing, which can occur as a result of cellular stress (e.g., oxidative stress) or during the early stages of apoptosis. These parameters could be measured further by biochemical methods to confirm the underlying molecular events associated with these morphological changes (e.g., free radical generation, lipid peroxidation, caspase activation, etc.).

### *Growth patterns*

Growth patterns may change as a result of exposure to toxin. Thus, the proportion of cells growing in colonies or singly would indicate changes in cell-cell interactions and potential changes in cell adhesion proteins, which could be targeted in subsequent molecular studies.

### *Metabolic assay*

#### *ATP levels*

ATP is an essential requirement for many energy-dependent processes and its levels can be affected by a variety of toxins. It can be used as a marker for cell viability as it is present in all metabolically active cells and its levels decline rapidly when cells undergo apoptosis or necrosis (Kangas *et al.*, 1984). A number of reagents and kits suitable for high throughput screening are available. Typically, the amount of ATP in cell lysates is determined by the light released from firefly luciferase-catalyzed oxidation of D-luciferin in the presence of ATP and oxygen. ATP and its major metabolites (ADP and AMP) in cell culture extracts can also be determined using an HPLC method (Yang and Gupta, 2003).

### *Dehydrogenase activity*

The activity of cellular dehydrogenases can be assayed by the reduction of the yellow methyl tetrazolium dye to its blue formazan reaction product. The reduced dye forms crystals in metabolically active cells and, after removal of growth medium, can be solubilized in an organic solvent such as dimethyl sulfoxide and quantified spectrophotometrically in microtiter plate format, for example in assays of *in vitro* toxicity of OPs (Hargreaves *et al.*, 2006).

Although metabolic assays such as those indicated above are not direct measurements of cell viability, a significant fall in either measurement is normally taken to indicate a fall in cell viability or cell number.

### *Calcium homeostasis*

Many toxins have a direct or indirect effect on mechanisms involved in the control of  $\text{Ca}^{2+}$  homeostasis. Such effects could be related to a breakdown in plasma membrane integrity, which would also be detected by other methods discussed previously (e.g., leakage of LDH), or it may be the result of a selective effect on  $\text{Ca}^{2+}$  channels or  $\text{Ca}^{2+}$  ATPases that regulate  $\text{Ca}^{2+}$  movements across cellular membranes. Changes in  $\text{Ca}^{2+}$  flux can be measured using a variety of  $\text{Ca}^{2+}$  binding dyes that fluoresce when bound to  $\text{Ca}^{2+}$  (e.g., Quin 2 and Fura 2). Detection requires spectrofluorimetric analysis which is potentially applicable to high throughput analysis of average change for a given cell population. However, analysis of changes in  $\text{Ca}^{2+}$  flux in individual cells requires a system that has an integrated microscope and image analysis software package.

### *Cell or tissue specific markers of toxicity*

This approach is useful for targeting key proteins or enzyme activities involved in the normal function(s) of the target cells or tissues. It is particularly useful in sub-lethal/ chronic studies of exposure to toxin, in order to determine whether a potentially reversible functional deficit occurs. The principal approaches used at the protein level are those of antibody-based detection and enzyme assays.

#### *Antibody-based detection*

This involves mainly immunoblotting techniques such as Western blotting and dot blotting coupled with densitometric analysis of antibody reactivity with target antigens. In Western blotting the cell extracts are normally denatured, separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose membrane filters (Towbin *et al.*, 1979) thus creating a replica of the gel pattern that is more accessible to antibody probes. By contrast, in dot blotting an extract is applied directly in a circular dot onto the membrane filter, without



chromatographic separation of proteins. Alternatively, experiments could be carried out on microtiter plates and the levels of antigens of interest determined in fixed cell monolayers by ELISA. Changes in the cellular or tissue distribution of antigens of interest can also be determined by immunohistochemical staining methods using enzyme-conjugated or fluorophore-conjugated antibody detection systems in a light microscope or fluorescence microscope, respectively. In the case of fluorescence microscopy quantification of antigen levels or distribution can be done with the aid of image analysis software. These approaches have been used effectively in mechanistic studies of OP toxicity (Hargreaves *et al.*, 2006).

#### Enzyme assays

Many cells and tissues can be monitored by the activity of enzymes that are known targets of the xenobiotics in question. For example, as discussed earlier, AChE activity is a good indicator of the acute neurotoxic effects of cholinesterase inhibitors such as OPs and carbamates *in vitro*, whereas NTE is a marker of the delayed neurodegenerative capability of OPs (Ehrich *et al.*, 1997). There are also enzymes that provide useful markers of specific cell types, such as tyrosine hydroxylase for dopaminergic neurons (Kuhn and Geddes, 2000), 2',3'-cyclic nucleotide 3'-phosphohydrolase for glial cells (McMorris, 1977) and alanine aminotransferase for hepatocytes (Amacher *et al.*, 1998). Where possible, changes in expression of the enzyme should also be taken into account when interpreting specific activity data.

#### Proteomic analysis

This approach can be useful in mechanistic studies of toxicity as it helps to identify novel protein markers of toxicity. Techniques of this type include the use of two-dimensional PAGE, chromatographic or protein chip fractionation of proteins from cell/tissue lysates or subcellular in order to identify changes in specific protein levels following toxin exposure. Proteins of interest are then normally digested with trypsin and the tryptic fragments are initially identified by "peptide mass fingerprinting" in a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer, followed by further confirmation using, e.g., tandem mass spectrometry (MS-MS) (Steen and Mann, 2004). Identification is facilitated by the use of freely available Internet-based gene and protein sequence databases such as MASCOT, SwissProt, etc.

#### Genomic analysis

Changes in the levels of specific proteins detected by proteome analysis could reflect changes in the regulation of protein turnover (i.e., how rapidly it is degraded

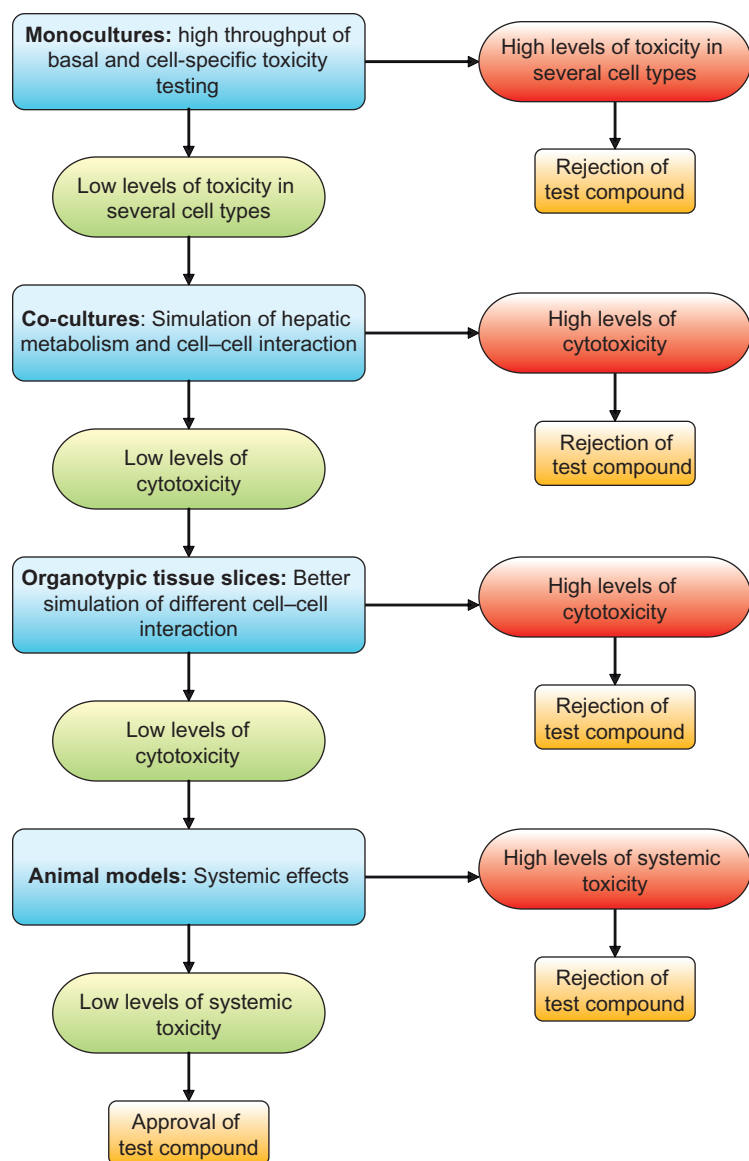
once synthesized) or changes in gene expression. Furthermore, depending on the cell or tissue fractionation procedures adopted, changes in low abundance proteins that could be functionally important may not necessarily be detected by proteomic methods. Changes in gene expression following exposure to toxin can be determined by a number of methods including reverse transcriptase polymerase chain reaction (RT-PCR) and DNA microarray analysis (Koizimo and Yamada, 2003).

RT-PCR is a targeted approach involving the production of complementary DNA (cDNA), by treatment of cell or tissue RNA with RT. The resultant cDNA is then incubated with primers (probes) that recognize specific genes or DNA sequences of interest, followed by amplification of such sequences by the Taq polymerase reaction. Changes in the level of amplified target sequence would indicate corresponding changes in the expression of the target gene to which the primers are matched. This approach is only semi-quantitative although accuracy can be improved by continuous measurement of amplicon levels using real-time RT-PCR. Levels of amplicon are normally compared to changes in household genes such as glyceraldehyde dehydrogenase, as an internal control.

A less targeted approach that, nevertheless, could help to identify novel changes in gene expression is that of DNA microarray analysis. In this technique, up to several thousand genes are immobilized on a template and probed with labeled RNA from control and treated cells or tissues. Image analysis software is then used to determine up- or down-regulation of genes due to toxin exposure. Controls usually involve averaged changes for a series of household genes. The researcher should be aware that a change in levels of gene expression does not necessarily indicate a corresponding change in protein levels or vice versa, as there are multiple levels of control. A multi-disciplinary approach is recommended to get an accurate overall picture of the chain of events following exposure to toxin.

## GENERAL SUMMARY AND CONCLUSIONS

A major issue facing toxicological science today is how to convert experimental data from *in vivo* and *in vitro* models into knowledge about molecular mechanisms of toxicity and safe levels of exposure to the agents tested. A number of animal models that have been used for many years for screening purposes have been gradually refined with increasing emphasis on improved experimental design, animal welfare and reduced animal use. More recent developments in the area of *in vitro* toxicity



**FIGURE 5.3** Flow chart showing principles of multi-tiered approach to toxicity testing.

testing have contributed significantly to the gradual reduction in animal use for toxicity screening and have helped to improve knowledge relating to mechanisms of cell- or organ-specific toxicity. Veterinarians have played an important role in the development of legislation and guidelines relating to the use and replacement of laboratory animals in toxicity assessment.

The generally accepted approach to screening compounds for toxicity is to use a multi-tiered system starting with simple monocultures of specific cell types, followed by co-cultures that simulate metabolic effects and/or cell-cell interactions in the whole organism, before carrying out final testing on animals (Figure 5.3). In this way, it is possible to eliminate compounds that exhibit high levels of basal toxicity in several cell types and reduce unnecessary suffering in animals. In both *in vitro* and *in vivo* testing it is important to use a battery of

appropriate endpoint measurements of both basal toxicity and cell/tissue-specific toxicity at each level, in order to increase the reliability and predictive potential of the data produced.

*In vitro* toxicology has made significant advances in the last few decades, by improving and finding solutions to its limitations. For example, cell culture systems are now being designed to be metabolically competent and viable for several weeks, making them appropriate not only for short- but also for long-term toxicity testing (Bhogal *et al.*, 2005). Extending this approach to a wider range of cell culture systems will improve the prospect of using *in vitro* methods for studying a wider range of chronic toxicological phenomena. However, further development and validation of *in vitro* models is required if they are to be adopted on a much wider scale in order to secure further reduction, replacement and

refinement of animal models. This will require further improvements in cell culture technology and growth conditions, such as the increased use of hollow fiber systems to facilitate continuous replenishment of media and supplements (Nussler *et al.*, 2001). The increased use of microsomal activation systems and/or established cell lines transfected with genes for mixed function oxidases is another approach that will help to increase the metabolic competence of *in vitro* models. Another promising *in vitro* approach involves the use of adult or embryonic stem cells of human or rodent origin. These cells can be either continuously cultured without being differentiated or they can generate a wide variety of functional mammalian cell types. In addition to its application in developmental toxicity testing, stem cell technology has been successfully used to study mechanisms of toxicity and to screen new pharmaceutical compounds for teratogenicity, genotoxicity, hepatotoxicity and cardiotoxicity (Davila *et al.*, 1998, 2004).

The development of medium to high throughput methods in proteomics, genomics and metabolomics, together with the revolution in bioinformatics, will lead to the accumulation of vast toxicological information, helping to elucidate mechanisms of toxicity in both *in vitro* and *in vivo* models (Bhogal *et al.*, 2005). These approaches will also help in the development and establishment of new biomarkers of effect or exposure.

In conclusion, although it is a desirable goal to replace all animal testing, animal-based toxicological testing is likely to continue for the foreseeable future. Further improvements in animal models will make them more reliable indicators of human toxicity, while the development of improved *in vitro* systems will eventually minimize the use of animals for toxicity studies. The veterinarian's role will therefore be to continue monitoring and improving the welfare of animals used in toxicity testing and to advise on issues relating to the development of *in vitro* alternatives.

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# Epidemiology of animal poisonings in the United States

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## INTRODUCTION

Although animals are exposed to potentially toxic agents on a daily basis, actual poisoning cases are uncommon when compared to other conditions of veterinary concern (infectious disease, trauma, metabolic disease, neoplasia, etc.). Clients will often present their animal with a suspicion of poisoning, only for the veterinarian to determine that the animal is suffering from an unrelated ailment. When evaluating information regarding suspected poisoning cases, it is important to consider the full exposure and patient history before determining whether a particular exposure is related to a clinical syndrome, as temporal coincidence does not necessarily equal causality.

The fundamental rule of toxicology as stated by Paracelsus, considered to be the “Father” of toxicology, is “the dose makes the poison.” Obviously the dose required to induce toxicosis will depend on a variety of factors, including the agent in question, species of animal exposed and route of exposure. Based on information from poison control centers, the majority of animal exposures to potentially toxic agents result in no signs developing (Hornfeldt and Murphy, 1992; Forrester and Stanley, 2004). However, clinically significant animal poisonings do occasionally occur from exposures to natural or man-made hazards. Knowledge of the most common features of animal poisonings can aid in instituting measures that may help to minimize exposures of animals to toxicants.

## BACKGROUND

The lack of a central reporting agency for animal poisonings makes epidemiological study difficult. With no mandated reporting, many suspected poisoning cases are managed by the attending veterinarian and forgotten. Confirmatory testing at veterinary diagnostic laboratories is not common, and no central mechanism exists for these laboratories to report their findings for epidemiological analysis. Most published information on animal poisonings has largely come from human or animal poison control centers that animal owners have contacted regarding potential exposures to toxic agents (Haliburton and Buck, 1983; Hornfeldt and Borys, 1985; Hornfeldt and Murphy, 1992, 1997, 1998; Forrester and Stanley, 2004) or from surveys of veterinary emergency centers or teaching hospitals (Osweiler, 1975; Cope *et al.*, 2006). Data from these sources can be helpful in determining trends and identifying emerging toxicants, but because of the frequent lack of complete historical information and confirmatory testing, care must be taken in the interpretation of each case of suspected poisoning.

Forrester and Stanley (2004) reported that exposures of animals to toxicants occurred more commonly in the summer, and this is consistent with data from the ASPCA Animal Poison Control Center’s AnTox™ database (Figure 6.1), although a peak in December, associated with the holiday season, is also present in the APCC data (ASPCA Animal Poison Control Center, unpublished data, 2010). The uptick in cases in the spring with

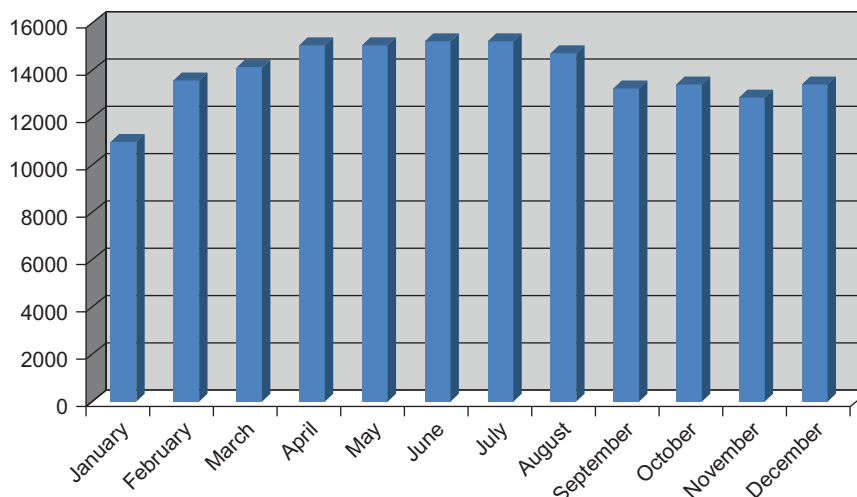


FIGURE 6.1 Monthly distribution of cases managed by ASPCA Animal Poison Control Center in 2010 (unpublished data).

peak cases during the summer is likely due to a variety of factors. Domestic animals tend to be more confined during the winter, particularly indoors, limiting their access to potentially toxic agents. Toxic plants and animals are not frequently encountered during the winter months, making exposures to these potentially poisonous entities unlikely. With the advent of warmer spring weather, domesticated animals have more access to outdoors and the plants, insects and other animals that reside there. The use of agents such as fertilizers, insecticides and herbicides also increases during these months, increasing the risk of animal exposures to these agents. Cool, wet, spring weather favors the rapid appearance of potentially toxic mushrooms, while hot summer weather can trigger growth of toxic blue-green algae in water. Other seasonal influences on the incidence of animal exposures to toxicants include increased presence of rodenticides in late fall as rodents begin to move indoors in response to cooling temperatures, increased use of psoriasis medication (e.g., calcipotriene) during winter months, increased use of ice-melting agents in winter, increased presence of lilies in the spring (Easter, Mother's Day), and increased presence of chocolate around the holidays of Valentine's Day, Easter, Halloween and the November–December holidays (Hautekeete, 2000; Gwaltney-Brant, 2001; Volmer, 2001; Merola, 2002).

The majority (>90%) of animal poisonings are accidental, acute in nature and occur near or at the animal owner's home (Hornfeldt and Murphy, 1992, 1998; Khan *et al.*, 1999). Malicious intent comprises less than 1% of all exposures to potentially toxic agents. The majority (70–95%) of exposures is due to acute ingestion, followed by acute dermal exposures (Hornfeldt and Murphy, 1992; Forrester and Stanley, 2004). Inhalation, envenomation, bites, ocular and parenteral routes of exposure

account for less than 1% and chronic exposures comprise approximately 1% of all reported cases. In 97% of exposures, a single agent is involved (Hornfeldt and Murphy, 1992, 1998).

## DEMOGRAPHICS

Based on data from human and animal poison control centers, dogs and cats are the species for which owners most frequently seek assistance with potential poisonings, accounting for 95–98% of all reported animal cases (Hornfeldt and Murphy, 1998; Xavier *et al.*, 2002; Forrester and Stanley, 2004; Giuliano Albo and Nebbia, 2004). Approximately 2–5% of reported animal poisoning cases involved other species of domesticated animals, exotic animals and wildlife. These percentages have changed considerably since 1983, when dogs and cats accounted for 44% of calls to an animal poison control center, with production animals (bovine, porcine, ovine) and equines making up 35% of calls (Trammel *et al.*, 1985).

## DOGS

Perhaps at least partly because of their inquisitive natures and willingness to investigate everything with their mouths, dogs far outrank other species when it comes to owners seeking aid for potential poisonings, making up 70–80% of all animal cases reported (Hornfeldt and Murphy, 1992, 1998; Xavier *et al.*, 2002; Forrester and Stanley, 2004; Berny *et al.*, 2009). There is

a tendency in the veterinary community to consider certain breeds, such as Labrador retrievers, to be more prone to exposure to potentially toxic agents. However, to verify such an impression, one must take into account the relative popularity of a particular breed. In other words: Are there more poisoning cases with Labrador retrievers because they really are more inclined to misadventure, or is it just that there are more Labrador retrievers in the population? A survey of the APCC database in 2005 (ASPCA Animal Poison Control Center, unpublished data, 2005) evaluated over 68,000 exposures of non-mixed breed dogs and compared the relative breed incidence to the 2005 Registration Statistics reported

by the American Kennel Club, the oldest and largest purebred dog registry in the United States (American Kennel Club (AKC), 2006). The results of this comparison are displayed in Table 6.1 and the top 20 breeds are compared graphically in Figure 6.2. In 2005, Labrador retrievers accounted for approximately 15% of AKC registered breeds, with almost three times more registered than the next most popular breed, the golden retriever. During the same year Labrador retrievers accounted for 17.5% of canine exposures in APCC cases, which was also three times more than the next most popular breed (the golden retriever) and which was not significantly different from the AKC statistics. In analyzing the data in

TABLE 6.1 Comparison of purebred dog breeds in relation to frequency of exposures to potentially toxic agents reported to the ASPCA Animal Poison Control Center with the relative popularity of the breed based on registration statistics from the American Kennel Club during 2005

	ASPCA (%)	AKC (%)	ASPCA rank	AKC rank		ASPCA (%)	AKC (%)	ASPCA rank	AKC rank
Labrador retriever	17.589	14.972	1	1	Soft coated wheaten terrier	0.530	0.219	39	59
Golden retriever	5.518	5.268	2	2	Chow Chow dog	0.517	0.182	40	64
German shepherd dog	4.206	4.889	3	4	Bulldog	0.601	2.232	41	13
Dachshund	3.711	4.188	4	6	Greyhound	0.485	0.015	42	134
Beagle dog	3.565	4.626	5	5	Pekingese dog	0.464	0.374	43	48
Chihuahua dog	3.535	2.560	6	11	Vizsla dog	0.379	0.387	44	44
Poodle	3.453	3.436	7	8	Newfoundland dog	0.377	0.379	45	46
Yorkshire terrier	3.193	5.130	8	2	Portuguese water dog	0.377	0.153	46	71
Boxer dog	3.150	4.047	9	7	Papillon dog	0.371	0.652	47	35
Cocker spaniel	3.012	1.775	10	15	Rhodesian ridgeback dog	0.360	0.263	48	54
Shih tzu dog	2.677	3.050	11	9	ShibaInu dog	0.333	0.174	49	66
Parson Russell terrier	2.647	0.145	12	72	American Eskimo dog	0.319	0.045	50	108
Miniature schnauzer dog	2.131	2.622	13	10	Airedale terrier	0.303	0.290	51	52
Pug dog	2.099	2.560	14	12	Collies	0.434	0.554	52	36
Bichon Frise dog	2.073	0.997	15	26	Bernese mountain dog	0.282	0.378	53	47
Maltese dog	1.829	1.485	16	19	Havanese dog	0.274	0.390	54	43
Pomeranian dog	1.718	2.119	17	14	Akita dog	0.272	0.308	55	51
Rottweiler dog	1.647	1.728	18	16	Mastiff dog	0.266	0.738	56	33
Siberian husky dog	1.566	1.026	19	25	Chinese Sharpei dog	0.264	0.385	57	45
Border collie dog	1.539	0.258	20	55	Chesapeake Bay retriever	0.256	0.362	58	49
Australian shepherd dog	1.331	0.676	21	34	Scottish terrier	0.252	0.412	59	40
Miniature pinscher dog	1.312	1.244	22	22	Wirehaired Fox terrier	0.249	0.124	60	76
Shetland sheepdog	1.120	1.551	23	18	English setter dog	0.246	0.071	61	97
Boston terrier	1.074	1.722	24	17	American Staffordshire terrier	0.245	0.182	62	63
West Highland white terrier	0.980	0.825	25	32	Italian greyhound	0.229	0.252	63	56
Weimaraner dog	0.844	0.936	26	29	Toy fox terrier	0.207	0.082	64	88
Basset hound	0.839	0.965	27	27	Silky terrier	0.208	0.175	65	65
Lhasa apso dog	0.795	0.426	28	39	French bulldog	0.205	0.457	66	38
Doberman pinscher dog	0.782	1.267	29	21	Samoyed dog	0.204	0.133	67	75
German shorthaired pointer	0.670	1.441	30	20	Alaskan malamute dog	0.195	0.223	68	58
Cavalier King Charles spaniel	0.667	0.834	31	31	Whippet dog	0.185	0.190	69	61
English springer spaniel	0.664	0.950	32	28	Welsh springer spaniel	0.179	0.028	70	124
Pembroke Welsh corgi dog	0.626	1.156	33	23	Pointer	0.178	0.042	71	110
Brittany dog	0.619	0.853	34	30	Bullmastiff dog	0.172	0.398	72	42
Australian cattle dog	0.614	0.156	35	70	Great Pyrenees dog	0.166	0.242	73	57
Dalmatian dog	0.578	0.113	36	77	Tibetan terrier	0.165	0.076	74	93
Cairn terrier	0.569	0.406	37	41	English Cocker spaniel	0.163	0.143	75	74
Great Dane dog	0.558	1.047	38	24	Saint Bernard dog	0.160	0.473	76	37
					Schipperke dog	0.156	0.108	77	80
					Bloodhound	0.150	0.338	78	50

(Continued)

TABLE 6.1 (Continued)

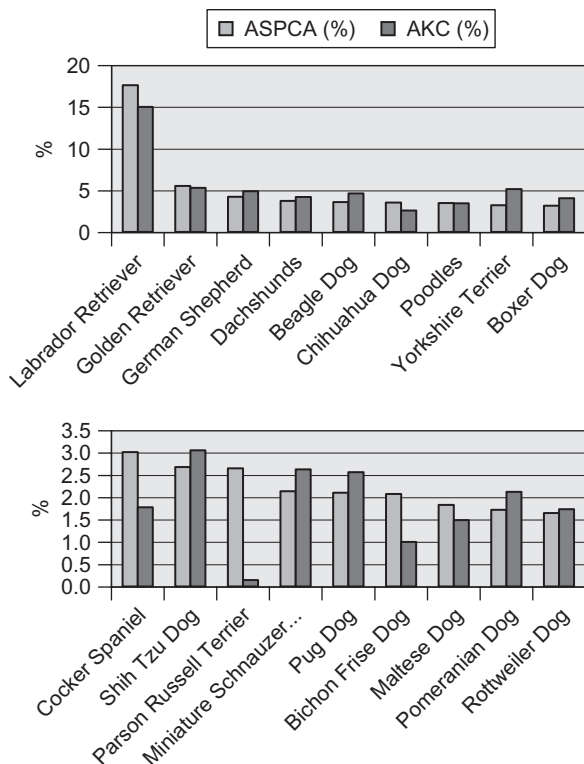
	ASPCA (%)	AKC (%)	ASPCA rank	AKC rank		ASPCA (%)	AKC (%)	ASPCA rank	AKC rank
Old English sheepdog	0.144	0.159	79	69	Australian terrier	0.031	0.045	115	107
Irish setter dog	0.132	0.165	80	67	Afghan hound	0.028	0.073	116	95
Welsh terrier	0.128	0.079	81	90	Tibetan spaniel	0.028	0.052	117	102
Standard Schnauzer dog	0.127	0.064	82	99	Pharaoh hound	0.023	0.012	118	140
Border terrier	0.124	0.103	83	82	Italian Spinoni dog	0.023	0.036	119	115
Chinese crested dog	0.122	0.281	84	53	Bedlington terrier	0.022	0.030	120	120
Keeshond dog	0.116	0.077	85	91	Lakeland terrier	0.022	0.019	121	129
Japanese Chin dog	0.108	0.161	86	68	Borzoi dog	0.020	0.076	122	94
Brussels griffon dog	0.106	0.196	87	60	Field spaniel	0.020	0.015	123	135
Flat coated retriever	0.100	0.064	88	98	Clumber spaniel	0.019	0.029	124	122
Basenji dog	0.096	0.107	89	81	Irish water spaniel	0.019	0.014	125	136
Staffordshire bull terrier	0.092	0.093	90	84	Kerry blue terrier	0.017	0.048	126	105
Cardigan Welsh corgi dog	0.090	0.111	91	79	Finnish Spitz dog	0.016	0.007	127	148
Irish terrier	0.084	0.034	92	117	Miniature bull terrier	0.015	0.023	128	126
Bull terrier	0.080	0.189	93	63	Wirehaired pointing	0.013	0.045	129	109
Belgian Malinois dog	0.079	0.085	94	87	Griffon dog				
Norwegian elkhound	0.071	0.073	95	96	Saluki dog	0.013	0.031	130	119
Bearded collie dog	0.070	0.053	96	101	Lowchen dog	0.013	0.012	131	139
Giant schnauzer dog	0.066	0.112	97	78	Briard dog	0.012	0.030	132	121
Belgian sheepdog	0.064	0.040	98	111	Puli dog	0.010	0.017	133	132
Norwich terrier	0.063	0.080	99	89	Affenpinscher dog	0.009	0.026	134	125
American foxhound	0.055	0.005	100	151	Polish sheepdog	0.009	0.013	135	138
Bouvier Des Flandres dog	0.054	0.092	101	85	Ibizan hound	0.009	0.017	136	133
Nova Scotia Duck Tolling retriever	0.054	0.038	102	113	German Pinscher dog	0.009	0.011	137	142
Gordon setter dog	0.054	0.088	103	86	Curly coated retriever	0.009	0.018	138	130
Black and tan Coonhound	0.052	0.017	104	131	Anatolian shepherd dog	0.009	0.037	139	114
Smooth fox terrier	0.052	0.052	105	103	American water spaniel	0.007	0.020	140	128
King Charles English toy spaniel	0.051	0.028	106	123	Komondor dog	0.006	0.008	141	145
Neapolitan Mastiff dog	0.049	0.034	107	116	English foxhound	0.006	0.002	142	154
Norfolk terrier	0.047	0.040	108	112	Sealyham terrier	0.004	0.008	143	146
Greater Swiss mountain dog	0.045	0.076	109	92	Harrier dog	0.004	0.005	144	153
Irish wolfhound	0.044	0.096	110	83	Dandie Dinmont terrier	0.004	0.006	145	149
Manchester terrier	0.044	0.050	111	104	Sussex spaniel	0.003	0.008	146	143
German wirehaired pointer	0.035	0.144	112	73	Scottish deerhound	0.003	0.021	147	127
Petit Basset Griffon Vendeen dog	0.035	0.031	113	118	Kuvasz dog	0.003	0.013	148	137
Belgian Tervuren dog	0.032	0.055	114	100	Glen of Imaal terrier	0.003	0.005	149	150
					Canaan dog	0.003	0.008	150	147
					Black Russian terrier	0.001	0.012	151	141

ASPCA Animal Poison Control Center, unpublished data (2005) and American Kennel Club (2006).

Table 6.1, a few breeds did appear to be over-represented in regards to exposures to potentially toxic agents. For instance, the Bichon Frise ranked 26th in AKC registrations yet ranked 15th in the APCC data, occurring in APCC cases (2.1%) at twice the incidence of AKC (1%); Welsh springer spaniels made up 0.03% of AKC registered dogs, but accounted for 0.18% of APCC dogs. However, care must be taken when evaluating the data from certain breeds (such as greyhounds, border collies and Parson Russell terriers), as many individuals of these breeds are registered with their own independent breed registry rather than the AKC; this may be one reason why all three of these dog breeds appear over-represented in APCC data.

Comparison of 2005 and 2010 APCC data shows that Labrador and golden retrievers still top the list of breeds involved in calls regarding potential poisonings in 2010 (ASPCA Animal Poison Control Center, unpublished data, 2005, 2010). Rounding out the top 10 breeds for 2010 poisoning calls, in descending order, are Chihuahua, Yorkshire terrier, Shih tzu, miniature Dachshund, Boxer, Beagle and American pit bull terrier. German shepherd dogs, which ranked 3rd in 2005, ranked 11th in 2010, although they were 2nd in 2010 AKC Registrations.

Previous reports of poisoning cases in dogs have indicated no gender predisposition, and most reports indicate that adults are most commonly involved, with an average age of approximately 4 years (63.6 years)



**FIGURE 6.2** Comparison of top twenty purebred dog breeds in relation to frequency of exposures to potentially toxic agents reported to the ASPCA Animal Poison Control Center with the relative popularity of the breed based on Registration Statistics from the American Kennel Club in 2010 (ASPCA Animal Poison Control Center, unpublished data, 2010).

(Khan *et al.*, 1999; Cope *et al.*, 2006). Given the wide age ranges in these studies (0.15–15 years) and the relatively low average age, it would appear that young adult dogs are most inclined to be exposed to potentially toxic agents. Reports of canine poisonings in Europe indicate that young dogs are at increased risk, with dogs 2 years of age and under comprising half or more of poisoning cases (Berny *et al.*, 2009).

## CATS

Due, perhaps, to their more discriminating habits and appetites, cats account for only 11–20% of reported animal exposures to potential toxicants, which is three times less frequent than dogs (Hornfeldt and Murphy, 1992, 1998; Xavier *et al.*, 2002; Forrester and Stanley, 2004, Berny *et al.*, 2009). In 2010, 71% of cats exposed to potential toxicants were identified as domestic (or American) shorthairs, 8% as domestic longhairs and 6% as domestic mediumhairs (ASPCA Animal Poison Control Center, unpublished data, 2010). Excluding these types of cats

due to the generic nature of their classification, the top 10 purebred cats were Siamese (24%), Main coon (15%), Persian (10%), Himalayan (8%), Ragdoll (6%), Bengal (6%), Russian blue (3%), Abyssinian (3%), Manx (2%) and Siberian (2%). Of these, the Persian, Maine coon, Siamese, Abyssinian and ragdoll are listed in the 10 most popular breeds registered by the Cat Fancier's Association in 2010 (CFA, 2010). As with dogs, no gender or age differences in incidence of exposure have been noted. Cats may, due to their grooming habits, be more susceptible to toxicants that come into contact with their fur; this is especially problematic with agents to which cats are exquisitely sensitive (e.g., ethylene glycol).

## OTHER SPECIES

Demographic information on potential poisonings in animal species other than dogs and cats in North America is largely lacking. Production animals are generally kept in large groups, meaning that when a toxicosis occurs, there is potential for multiple animals of varying age and genders to be exposed. Most production animals are kept in some form of confinement, which limits the potential for exposure to toxic agents. However, mistakes in management, such as feed mixing errors or improper ventilation, may result in acute or chronic toxicosis in large numbers of animals. A study of livestock poisonings in Greece indicated that sheep were the commonly poisoned species, attributed primarily to their ingestion of potentially contaminated soil (Guitart *et al.*, 2010a).

Wildlife is almost continuously exposed to toxic agents in the environment, but specific demographic information is not available in most cases. In France, birds are reported to be more commonly poisoned than mammals (Guitart *et al.*, 2010b). Seasonality of poisoning incidences in wildlife would vary with the agent involved. For instance, avian botulism in waterfowl tends to be seasonal, with most cases occurring between the months of July and September (Locke and Friend, 1989). In addition to naturally occurring toxicants, exposures to man-made hazards (e.g., oil spills, industrial effluents) can result in significant wildlife morbidity and mortality.

## AGENTS INVOLVED

There is an unlimited number of agents by which exposed animals may become poisoned, and for the most part, which specific agents are involved in animal poisonings will be dependent upon what is available in the animals' environment, the potential or inclination



**TABLE 6.2** Exposures of animals to various toxic agents reported by veterinary clinics and poison control centers, 1975–2006

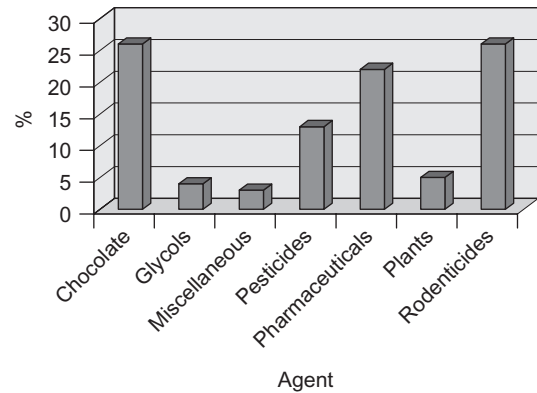
Agent	1975	1978–1981	1990	1993	2006
Chocolate	NR	NR	NR	NR	26%
Glycols	1%	NR	10%	NR	4%
Metals	2%	7%	1%	NR	0%
Miscellaneous	47%	56%	17%	34%	3%
Pesticides <sup>++</sup>	23%	20%	21%	21%	13%
Pharmaceuticals	NR	10%	25%	24%	22%
Plants	1%	12%	2%	10%	5%
Rodenticides	27%	7%	14%	7%	26%

NR: not reported; ++: combined insecticide and herbicide exposures. Osweiler (1975), Haliburton and Buck (1983), Hornfeldt and Murphy (1992, 1998) and Cope *et al.* (2006).

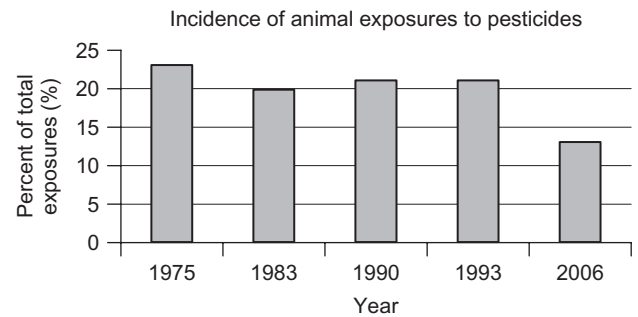
for the animal to be exposed to the agent, the amount of agent to which the animal is exposed, and the individual sensitivity of the animal to the effects of the agent. The potential for exposure to specific agents may be uniform throughout the year or may be seasonal, depending on the agent and the species involved. For instance, exposures to lawn care products, such as herbicides and insecticides, would be expected to be more common during the seasons when these products are most in use (i.e., spring and summer).

Table 6.2 summarizes the most common agents involved in animal exposures reported to veterinary emergency referral centers and human or animal poison control centers in the United States. These exposures predominantly involve companion animals, particularly dogs and cats, and are consistent with the types of animal exposures reported by poison control centers in other countries (Xavier *et al.*, 2002; Giuliano Albo and Nebbia, 2004).

Rodenticides, pharmaceuticals and chocolate make up the majority of agents in the most recent report summarized in Figure 6.3 (Cope *et al.*, 2006). Rodenticides and chocolate made up approximately one quarter of all exposures, followed by pharmaceutical agents, which accounted for 22% of exposures. Pharmaceutical exposures have increased since first reports in 1983 (Beasley and Trammel, 1994), likely due to the increased use of these agents in veterinary and human medicine over the past 20 years. The most common pharmaceutical agents associated with animal exposures are analgesics (primarily non-steroidal anti-inflammatory drugs) and central nervous system drugs (sedatives, antipsychotics, stimulants, etc.) (Hornfeldt and Murphy, 1992, 1998). None of the reported data from poison control centers included information specifically on chocolate cases, but a veterinary emergency hospital reported that exposure of dogs to chocolate accounts for one in every four presentations for potential toxicosis (Cope *et al.*, 2006). Pesticides have historically been responsible for large numbers of exposures and toxicoses in domestic animals (Beasley and



**FIGURE 6.3** Most common agents involved in canine exposures presenting to a veterinary emergency center in 2006 (Cope *et al.*, 2006).



**FIGURE 6.4** Comparison of incidence of animal exposures to pesticides reported to human poison control centers and veterinary clinics (Osweiler, 1975; Haliburton and Buck, 1983; Trammel *et al.*, 1985; Hornfeldt and Murphy, 1992).

Trammel, 1994), but the incidence appears to be declining (Figure 6.4). This may be due in part to the development of newer herbicides and insecticides that have a much higher margin of safety in mammals than those used in the past, as well as perhaps better public awareness of the potential hazards of these agents to pets.

Less information is available regarding exposure of non-canine and non-feline animals to potential toxicants. For livestock, plant poisoning causes tremendous economic losses to producers, estimated to exceed \$350 million per year (Galey, 1996). Losses from poisonous plants are due to deaths as well as loss of productivity. Other agents reported to be responsible for significant livestock loss include mycotoxins, organophosphate and carbamate insecticides, nitrate and lead, although lead poisoning appears to be on the decline in livestock. For waterfowl, avian botulism is a concern, resulting in the loss of thousands to millions of birds each year (Locke and Friend, 1989). Lead toxicosis was once a significant cause of loss of waterfowl in the United States due to ingestion of lead shot left by hunters, but the incidence of lead toxicosis has decreased following an enforced

ban on the use of lead shot on waterfowl (Gwaltney-Brant, 2004). However, lead intoxication still occurs in raptors that ingest upland prey that have shot embedded in their tissues. Wild animals ingesting the tissues of animals that have been euthanized with barbiturates are at risk of toxicosis (Hayes, 1988), and toxicosis has been reported in a variety of species feeding on animals intentionally poisoned with pesticides (Stroud, 1998; Wobeser *et al.*, 2004). The use of non-steroidal anti-inflammatory drugs in livestock has been linked to the marked decline in vultures and other scavenging birds in India, Asia and Africa (Naidoo *et al.*, 2009, 2010). Intentional poisoning of wildlife with pesticides is most commonly accomplished using carbofuran and aldicarb, while accidental or secondary poisoning of wildlife by pesticides most often involves strychnine, famphur, fenthion and avitrol (Stroud, 1998). Oil spills near sea shores have resulted in significant loss of life of animals living in or around these areas, and industrial pollution from agricultural or industrial effluents has similarly caused deaths in a variety of wildlife species.

## SIGNS AND OUTCOMES

Based on poison control center statistics, the majority (57–63%) of animal exposures to potential toxicants result in no signs for the patient, due either to insufficient level of exposure or successful decontamination by animal caretakers (Figure 6.5). Mild signs developed in 25–27% of animal exposures to suspected toxicants, moderate signs developed in 7–8%, major signs developed in approximately 1% and death occurred in 2–3% of cases where outcomes were known (Hornfeldt and Murphy, 1992, 1998). Mild signs are those in which some clinical signs developed but are expected to be mild and self-limiting and not in need of treatment (e.g., hypersalivation, mild vomiting, etc.). Moderate signs are those in which signs that develop are more pronounced, prolonged or of a systemic nature and merit some form of treatment, although the signs would not be expected to be life-threatening (e.g., protracted vomiting, severe diarrhea, mild to moderate hypotension). No residual effects would be expected following recovery. Major signs are those in which life-threatening conditions exist or there is potential for significant residual disability or disfigurement following recovery from the acute episode (e.g. seizures, renal injury, etc.).

Agents most frequently reported to be associated with mild to moderate signs in animals are pharmaceuticals (19%), pesticides (insecticides and herbicides, 14%), plants (10%), cleaning products (8%) and mushrooms (3%) (Hornfeldt and Murphy, 1998). Agents associated with major signs included pesticides (45%),

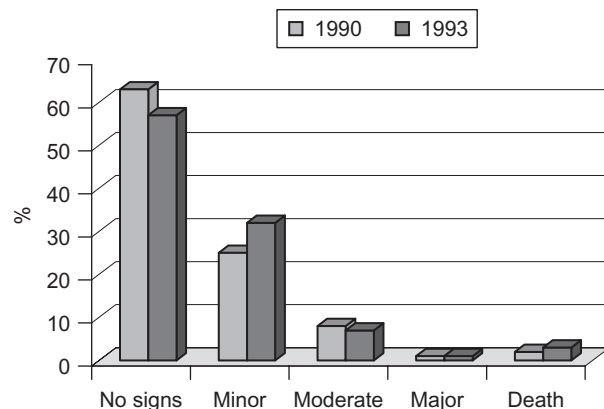


FIGURE 6.5 Degree of illness in animals following exposure to potentially toxic agents (Hornfeldt and Murphy, 1992, 1998).

pharmaceuticals (25%), plants (15%), rodenticides (9%) and cleaning products (8%). Deaths were most commonly associated with exposures to pesticides (24%), pharmaceuticals (17%), rodenticides (16%), plants (9%), automotive products (predominantly ethylene glycol, 9%) and cleaning products (8%). Organophosphate insecticides, ethylene glycol and long-acting anticoagulant rodenticides were the top three agents responsible for deaths in animals.

## CONCLUSIONS

Poisonings are a serious cause of morbidity and mortality in animals, particularly in dogs and cats. Based on information obtained by veterinary clinics and human and animal poison control centers the incidence of animal poisoning does not appear to be waning, although the agents to which animals are exposed do change with time. While significant pesticide exposures may be on the decline due to the development of newer and less toxic pesticides, exposures to other agents, such as chocolate and pharmaceutical agents, may be on the rise. Knowing what agents have the potential to be involved in serious toxicoses should allow veterinarians to better educate their clients on means of preventing animal poisonings through the appropriate use of household products and the removal of potential hazards from the animals' environments.

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# Epidemiology of animal poisonings in Europe

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## INTRODUCTION

A centralized veterinary poison control/information center does not exist in the EU. In many countries information related to animal poisoning is unavailable (BG, CY, EE, HU, LT, LV, MT, PT, RO, SI, SK) or inadequate (DK, LU) and refers only to isolated case reports when it does exist. Occasionally, epidemiological data on animal poisoning gathered by universities, research institutes, government institutions or independent laboratories has been published in the last decade in AT, DE, EL, ES and IT (see [Table 7.1](#) for country abbreviations).

In certain countries such as CZ, EL, ES, FI, IE, IT, NL, PL and SE, human poison control centers collect data on animals also. In IT, the Servizio di Assistenza Tossicologica Veterinaria (SATV) used to provide telephone assistance in cases of small animals only ([Giuliano Albo and Nebbia, 2004](#)), whereas the Assistenza Tossicologica Veterinaria (ATV) is now in operation. In BE, the Belgian National Poison Center in Brussels (BPC) holds a 24-hour hotline for suspected human and animal poisoning. The Laboratory of Toxicology of Ghent University (LTGU) and the Centre d'Informations Vétérinaires en Pharmacotoxicologie, Université de Liège, are specialized in analytical confirmation of animal poisoning for the northern and southern parts of BE, respectively ([Vandenbroucke et al., 2010](#)).

Centers in the EU exclusively dedicated to animal poisoning are located in FR and in the UK. In FR, the College of Veterinary Medicine of Lyon (CNITV) offers 24-hour specialized assistance for suspected cases of animal poisoning and receives between 12,000 and

14,000 calls each year, while the corresponding toxicology laboratory analyses between 1500 and 2000 samples. A second center is located at the College of Veterinary Medicine of Nantes (CAPAE-Ouest) which is open during regular business hours ([Berny, 2007; Berny et al., 2010a](#)). In the UK, the Veterinary Poisons Information Service (VPIS) provides services only to veterinarians, whereas the Royal Society for the Prevention of Cruelty to Animals (RSPCA) records suspected cases of deliberate acts reported by pet owners. In cases where there is hard evidence of pesticide poisoning including sporadic incidents involving livestock and pets, surveillance is conducted by the Veterinary Laboratories Agency (VLA) for production animals and by the Wildlife Incident Investigation Scheme (WIIS) for wildlife.

## SPECIES INVOLVED

In numerous EU countries such as BE, DK, ES, FR, IT, PL, SE and the UK, the species most commonly involved in poisoning incidents is the dog followed by the cat ([Amorena et al., 2004; Giuliano Albo and Nebbia, 2004; DEFRA, 2007; Berny et al., 2010a; Vandenbroucke et al., 2010](#)). With respect to analytical investigations at the CNITV and LTGU laboratories ([Figure 7.1](#)), the resulting numbers are slightly different due to the significant number of wildlife disease surveillance plans, resulting in a substantial number of baits being included in the analyses (22% of cases submitted annually at LTGU).

TABLE 7.1 Abbreviations used in the text for the 27 European Union (EU) countries

AT	Austria
BE	Belgium
BG	Bulgaria
CY	Cyprus
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
EL	Greece
ES	Spain
FI	Finland
FR	France
HU	Hungary
IE	Ireland
IT	Italy
LT	Lithuania
LU	Luxembourg
LV	Latvia
MT	Malta
NL	Netherlands
PL	Poland
PT	Portugal
RO	Romania
SE	Sweden
SI	Slovenia
SK	Slovakia
UK	United Kingdom

Among farm animals, most of the poisoning incidents involve cattle (VIDA, 2009; Guitart *et al.*, 2010a; Vandenbroucke *et al.*, 2010) followed by sheep and goats.

The last two are also the most common species involved in poisoning incidents in EL (Roubies *et al.*, 2008; Guitart *et al.*, 2010a). Generally, poultry and pigs are rarely involved in poisoning episodes and minor percentages are also reported for exotic animals and rabbits, often included in the group “other species.”

As far as wildlife, birds, especially waterfowl and raptors, are more commonly reported than mammals as victims of poisoning (Samouris *et al.*, 2007; Guitart *et al.*, 2010b). Apart from specific toxic environmental disasters, deliberate primary or secondary poisoning incidents are of concern in all EU countries and mainly involve birds of prey such as common buzzards and red kites (DEFRA, 2007; Guitart *et al.*, 2010b; Vandenbroucke *et al.*, 2010). Indeed, the proportion of wildlife cases at the CNITV diagnostic laboratory, a part of the national wildlife disease surveillance network (SAGIR), is very high (Berny, 2007). As this network is supported financially by hunters, it mostly deals with game species but an increasing number of cases regarding protected and endangered species has also been recorded (Berny and Gaillet, 2008). A Spanish national program (*Programa Antídoto*) reported several cases of deliberate poisoning of wild birds from 2006 to 2009. During this period, 243 griffon vultures, 42 red kites and 21 Cinereous vultures were found dead, among other wild birds. Official data from the Ministry of the Environment in 2008 indicated that in ES some 7000 members of protected wild species had been poisoned in the previous 15 years.

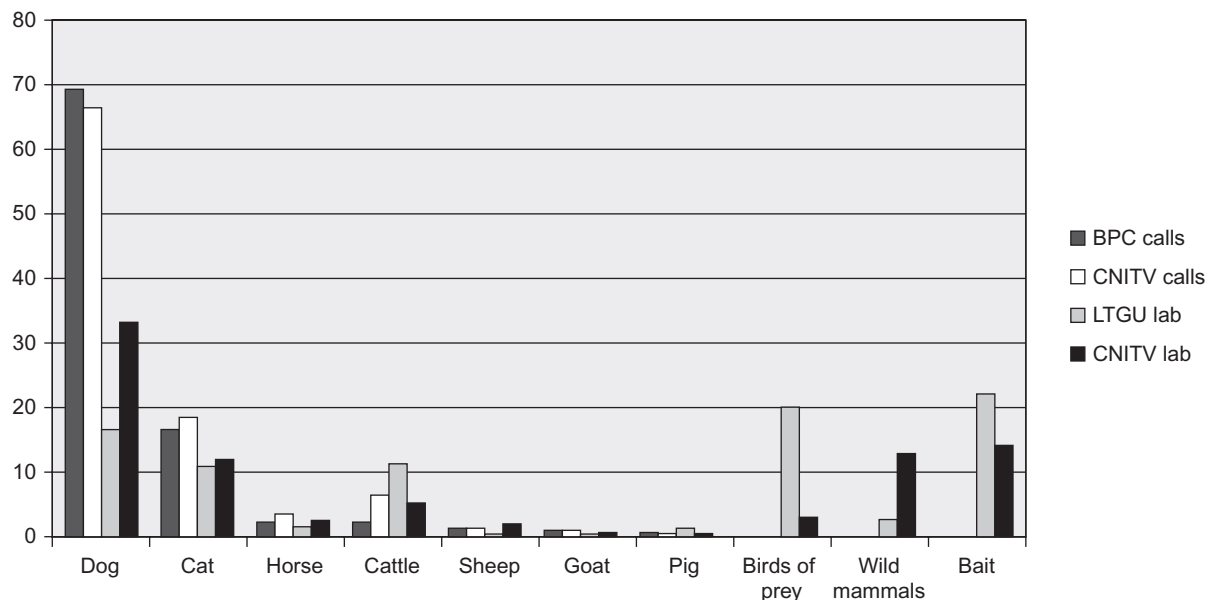
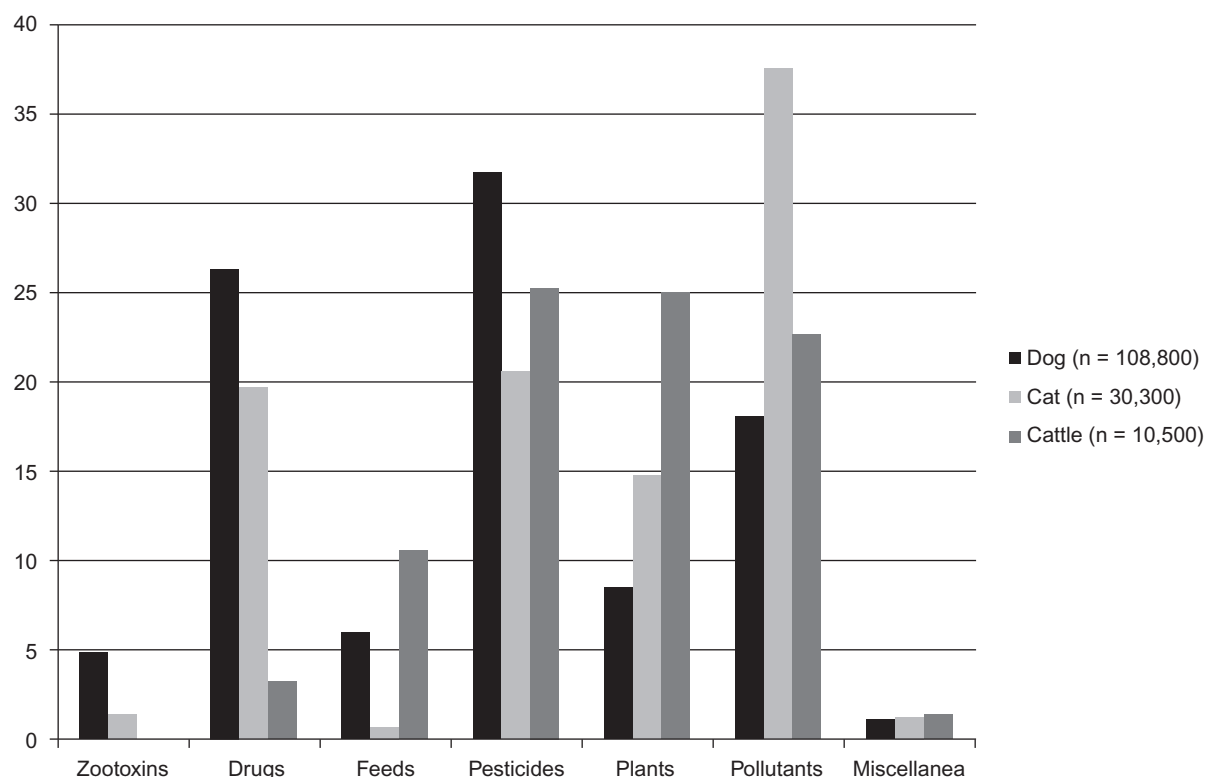


FIGURE 7.1 Distribution of species (including bait) in poisoning cases at the Belgian Poison Center (BPC calls,  $n = 2,300$  calls annually), the French Animal Poison Control Center (CNITV calls,  $n = 163,883$  from 2000 to 2010), the Belgian Laboratory of Toxicology of the Faculty of Veterinary Medicine from Ghent University (LTGU lab,  $n = 233$  cases annually) and the French College of Veterinary Medicine Diagnostic Toxicology Laboratory in Lyon (CNITV lab,  $n = 16,348$  cases from 2000 to 2010).





**FIGURE 7.2** Toxic classes involved in suspected poisoning cases at the French College of Veterinary Medicine CNITV in Lyon (% in dogs, cats and cattle, from 1991 to 2008).

## TOXIC CLASSES

In most species, pesticides are one of the main causes of poisoning. Statistical data from CNITV confirms this (Figure 7.2), as it is only exceeded by a big group of pollutants (metals, detergents, fertilizers, etc.). Among pets, insecticides, rodenticides, herbicides, fungicides and other pest control substances, used either for plant protection or for indoor pest control, are frequently involved in deliberate or accidental poisoning episodes (Berny *et al.*, 2010a). Drugs are a common source of poisoning for companion animals, caused by the owner's negligence and off-label use of drugs (Modra and Svobodova, 2009; Berny *et al.*, 2010a). Other poisons include household products, plants, fuels, ethylene glycol, zootoxins, cosmetics and drugs of abuse (cocaine or cannabis) (Berny *et al.*, 2010a). For horses, plant poisoning is most frequently reported (Berny *et al.*, 2010a).

In food-producing animals, pesticides are again the most common class of suspected toxicants (Guitart *et al.*, 2010a). In FR, the most common culprits are mainly insecticides and seed-coated products followed by drugs, plants and nutritional disorders resulting from the improper use of foods and feeds and unbalanced diets (e.g., urea poisoning, grain overload). Other common toxicoses involving cattle and pigs result from

plants and metals in ruminants and mycotoxins (Guitart *et al.*, 2010a). In EL, chronic Cu poisoning appears to be a rather common problem mainly in sheep husbandry (Roubies *et al.*, 2008).

For wildlife, pesticides (mainly anticholinesterase and anticoagulant agents) and metals (particularly Pb) are the common causes of poisoning (DEFRA, 2007; Samouris *et al.*, 2007; Mateo, 2009; Modra and Svobodova, 2009; Guitart *et al.*, 2010b). Cyanobacteria toxicoses and botulism involving waterfowl have also been reported (Modra and Svobodova, 2009; Guitart *et al.*, 2010b).

## NON-METALS AND METALLOIDS

Fluorosis in the EU has been observed following the intake of borehole water with high levels of F in region-specific deep-lying clay layers, as reported for example in the case of turkeys (Berny *et al.*, 2010a). A particular incident was reported in BE involving cattle, following the use of calcium sulfate ( $\text{CaSO}_4$ ) as a binder agent in beet pulp resulting in a higher than normal level of F (Vandenbroucke *et al.*, 2010). Poisoning with As predominantly involved cattle, but rarely sheep (Sharpe and

Livesey, 2005; Vandenbroucke *et al.*, 2010). Se toxicoses are also rare but two cases have been reported involving sheep in the UK (Sharpe and Livesey, 2005) and pigs in ES (Casteignau *et al.*, 2006).

## METALS

Telephone calls to EU poison centers, however, do not give us the full picture of animal poisoning. Probably more than any other single toxic agent, certain metals, especially Pb in the case of wild birds, still continue to cause numerous suspected and confirmed animal poisoning cases in the EU.

### Copper

Chronic Cu poisoning is a fairly common clinical problem in the Greek sheep industry (Roubies *et al.*, 2008; Guitart *et al.*, 2010a). Field investigation of one case in EL revealed the source of Cu to be a heap of litter from a broiler farm to which the sheep had accidental access. In the UK, three outbreaks of Cu poisoning which killed at least 55 ewes were detected in 2005 and 2006, associated with organic farming and the consumption of red clover (*Trifolium pratense*) and white clover (*T. repens*). In BE, excessive amounts of Cu in commercially prepared milk replacers caused chronic toxicity in veal calves in two different farms (Vandenbroucke *et al.*, 2010).

### Lead

In a Spanish farmland property located only several meters from a battery-recycling site that had been in operation for 20 years, the death of seven horses and one donkey was documented due to Pb poisoning. Unusual findings have been reported involving horses: a 13-year-old 700 kg Irish Draught cross gelding presenting abnormal respiratory noise during exercise caused by bilateral recurrent laryngeal nerve dysfunction, was diagnosed in the UK subsequent to Pb toxicosis (Allen, 2010).

Pb poisoning is very common for large animals, especially for cattle and sheep. In the UK, more than 450 Pb incidents were investigated by the VLA between 1998 and 2008, mostly related to discarded batteries, old paint and geochemical sources (Payne and Livesey, 2010). In AT, BE, ES and FR, lethal cases involving cattle have also been reported due to the ingestion of fragments of discarded batteries or the contamination of pastures with ash residue from illegal bonfires (Krametter-Froetscher *et al.*, 2007; Guitart *et al.*, 2010a).

In the case of birds, there is extensive literature in the EU documenting poisoning episodes due to the accidental ingestion of small Pb objects, namely spent ammunition and lost fishing weights. This kind of poisoning has long been recognized as a major cause of death in several species (Fisher *et al.*, 2006; Mateo, 2009; Pain *et al.*, 2009). In ES, waterfowl mortality rates were conservatively estimated to be 50,000 a year at the end of the last century, just before the implementation of a ban on the use of Pb shot in the wetlands of this country. Up until 2008, only about half of the EU countries had taken some type of restrictive action against the introduction of Pb through hunting in the marshlands and only four countries (BE, DK, the NL and SE) totally or partially extended this measure to other zones (Thomas and Guitart, 2010). It is worth mentioning that substantial amounts of metallic Pb continue to remain in wetlands, riverine and terrestrial ecosystems for decades or centuries and this represents a source of continued risk of bird poisoning.

The number of hunters and shooters in the entire EU has been estimated to be about 9,000,000 and the Pb ammunition fired yearly about 40,000 t (Thomas and Guitart, 2010). Very high concentrations of Pb shot (>150 per m<sup>2</sup> in the first 10–20 cm of depth) have been reported in some EU wetlands. Consequently a high prevalence of Pb shot ingestion was found, although the intensity varied among bird species and among regions across the EU. Remarkably, Mateo (2009) detected a clear negative relationship between the prevalence of Pb shot ingestion and the wintering population trend in Europe of 15 waterfowl species. Poisoning of swans after ingestion of Pb split-shot or sinkers also occurs in IE and the UK (Perrins *et al.*, 2003). Non-raptor upland birds are also victims and a prevalence of 1.4% and 7.8% for red-legged partridges was found in the UK and ES, respectively.

There have been several reports over the last 10 years of Pb poisoning or high levels of exposure to Pb (compatible with sub-clinical or clinical signs) involving birds of prey (see Table 7.2), both predators and scavengers (Fisher *et al.*, 2006; Mateo *et al.*, 2009; Pain *et al.*, 2009; Guitart *et al.*, 2010b). Raptors are at risk both from prey killed with shotguns, such as wildfowl, upland game birds and rabbits, and with rifles, such as deer and other large game. Recent radiographic studies have shown that many small bullet fragments remain inside prey shot with Pb-based bullets, posing a risk not only for raptors but also for human consumers of this hunted game. Up until 2009, Pb poisoning had been reported in 17 species of birds of prey in Europe, some of which are near-threatened such as the white-tailed eagle or are vulnerable such as the Spanish imperial eagle (Mateo, 2009).

Last but not the least, a case of Pb poisoning involving zoo dolphins was reported in NL in 2008. Apparently, the animals had ingested metal from a damaged diving belt.

TABLE 7.2 Poisonings reported in the last decade in the EU for wildlife species

Species	Poison	Country	Species	Poison	Country
American mink ( <i>Mustela vison</i> )	Pesticides (anticoagulants)	FR	Greylag goose ( <i>Anser anser</i> )	Metals (Pb)	ES
Atlantic puffin ( <i>Fratercula artica</i> )	Oil	ES, FR, PT	Golden eagle ( <i>Aquila chrysaetos</i> )	Metals (Pb)	AT, DE
Barn owl ( <i>Tyto alba</i> )	Metals (Cd)	ES	Griffon vulture ( <i>Gyps fulvus</i> )	Pesticides (anticholinesterases)	ES, EL
Bearded vulture ( <i>Gypaetus barbatus</i> )	Pesticides (anticoagulants)	UK	Herring gull ( <i>Larus argentatus</i> )	Toxins (botulism)	SE
Cinereous vulture ( <i>Aegypius monachus</i> )	Metals (Pb)	ES, FR	Honey bee ( <i>Apis mellifera</i> )	Pesticides	UK
Common buzzard ( <i>Buteo buteo</i> )	Pesticides (strychnine, carbamates)	EL, ES	Marsh harrier ( <i>Circus aureoginosus</i> )	Metals (Hg)	IT
Common guillemot ( <i>Uria aalga</i> )	Metals (Pb)	ES	Mute swan ( <i>Cygnus olor</i> )	Pesticides (carbamates)	CZ
Common kestrel ( <i>Falco tinnunculus</i> )	Oil	ES	Northern gannet ( <i>Sula bassana</i> )	Metals (Pb)	IE, UK
Common raven ( <i>Corvus corax</i> )	Pesticides (carbamates)	BE, CZ	Northern goshawk ( <i>Accipiter gentilis</i> )	Oil	ES, FR, PT
Common scoter ( <i>Melanitta nigra</i> )	Oil	ES, FR, PT	Otter ( <i>Lutra lutra</i> )	Metals (Pb)	DE
Common vole ( <i>Microtus arvalis</i> )	Oil	ES	Peregrine falcon ( <i>Falco peregrinus</i> )	Pesticides (carbamates)	CZ
Common wood pigeon ( <i>Columba palumbus</i> )	Pesticides (carbamates)	CZ	Polecat ( <i>Mustela putorius</i> )	Oil (polycyclic aromatic hydrocarbons)	ES
Dolphin	Pesticides (carbamates)	FR	Razorbill ( <i>Alca torda</i> )	Pesticides (anticoagulants)	FR
Egyptian vulture ( <i>Neophron percnopterus</i> )	Oil	FR	Red kite ( <i>Milvus milvus</i> )	Oil	ES, FR, PT
Eleonora's falcon ( <i>Falco eleonora</i> )	Pesticides (carbamates)	CZ		Metals (Pb)	ES, FR, UK
Eurasian eagle-owl ( <i>Bubo bubo</i> )	Pesticides (carbamates)	FR		Pesticides (anticholinesterases, rodenticides)	FR
European hare ( <i>Lepus europaeus</i> )	Metals (Pb)	NL	Rock pigeon ( <i>Columba livia</i> )	Metals (Pb)	BE
European rabbit ( <i>Oryctolagus cuniculus</i> )	Metals (Pb)	ES	Rough-legged buzzard ( <i>Buteo lagopus</i> )	Pesticides (carbamates)	CZ
Fallow deer ( <i>Dama dama</i> )	Pesticides (carbamates)	ES	Spanish imperial eagle ( <i>Aquila adalberti</i> )	Metals (Pb)	ES
Ferret ( <i>Mustela putorius furo</i> )	Pesticides (anticoagulants)	FR	Waterbirds (several species)	Metals (Pb)	BE, ES, FR, IT, PT
Greater flamingo ( <i>Phoenicopterus ruber</i> )	Pesticides (anticoagulants)	FR		Toxins (cyanobacteria)	ES
	Drugs of abuse (cannabis, hashish)	NL	White-tailed sea eagle ( <i>Haliaeetus albicilla</i> )	Metals (Pb)	AT, DE, FI, SE
	Metals (Pb)	ES, IT		Pesticides (carbamates)	DE, CZ
	Toxins (cyanobacteria)	ES	Wild boar ( <i>Sus scrofa</i> )	Pesticides (carbamates, rodenticides)	CZ, FR

## Other metals and minerals

Mercury (Hg) poisoning is uncommon in domestic animals but should be taken into account as a potential cause of renal failure. In DE, a 4-month-old male German shepherd dog presented with a 2-day history of vomiting, bloody diarrhea and severe renal function impairment. After euthanasia due to the poor prognosis, the owner admitted that a barometer containing Hg had broken in the bedroom about a week before the dog had developed clinical signs. The route of exposure to Hg was presumed to be digestive and respiratory (Hansmann *et al.*, 2009).

In FI, clinical thermometers ranked as one of the top 10 substances involved in dog poisoning inquiries from 2001 to 2003. In IT, Hg exposure following the accidental ingestion of thermometers accounted for more than one third of the total calls regarding metal poisoning in dogs (Giuliano Albo and Nebbia, 2004). Also in IT, a marsh harrier was found dead in a wetland in the north-east and a postmortem diagnosis of Hg poisoning was established, based on levels of  $20\mu\text{g g}^{-1}$  in both liver and kidney.

In NL, an accident took place when a veterinarian advised the owner of a 4.5kg Schipperke dog to give it table salt to induce vomiting after ingestion of one tablet of digoxin. Apparently, the dose administered

was too large and the dog presented neurological signs of salt poisoning and died a few hours later. A similar case occurred in FR with a 5-year-old female Doberman pinscher (Pouzot *et al.*, 2007), but this time it was treated successfully and survived.

Zn toxicity is rarely observed in the EU. Two recent cases involving dogs were reported in the UK when both animals ingested a badly designed tag distributed by an international animal health laboratory to identify that the dogs had been microchipped (Adam *et al.*, 2011). In IT, there was a case of a puppy which died after the ingestion of a pipe fragment from the domestic heating system (Gandini *et al.*, 2002). Unusual clinicopathological findings have also been reported, as in the case of a 6-year-old female Labrador retriever that had ingested a Zn toy; hematological findings revealed a large number of Heinz bodies and marked anemia (Bexfield *et al.*, 2007).

## PESTICIDES

Pesticides are common culprits in animal poisoning cases, as a result of inappropriate or careless use. For companion animals, most of the suspected poisoning cases in BE, EL, ES and FR involved anticholinesterase insecticides mainly carbamates followed by organophosphates (Berny *et al.*, 2010a; Vandenbroucke *et al.*, 2010). In ES, rodenticides are as much to blame as insecticides, whereas in IT the most common cause of pet toxicosis is rodenticides (Berny *et al.*, 2010a). A 6-year retrospective study in AT investigated pesticide poisoning and revealed 123 positive cases; out of 225, 47.2% involved dogs and 34.1% cats (Wang *et al.*, 2007). The highest percentage of incidents was associated with carbamates (50.3%), followed by herbicides and molluscicides (22.3%), anticoagulant rodenticides (18.9%), organophosphates (5.1%) and nonanticoagulant rodenticides (3.4%) (Wang *et al.*, 2007).

Grazing animals are more exposed to pesticides than livestock kept in the barn. Nevertheless, application or storage of pesticides in barns occasionally leads to accidental animal toxicosis. In certain EU countries such as EL and FR, pesticide poisoning is, by far, the most common cause of livestock toxicosis in comparison to other countries such as BE, ES and IT (Guitart *et al.*, 2010a).

Pesticides and more specific anticholinesterase insecticides and rodenticides followed by molluscicides and avicides are among the most frequently reported causes of wildlife poisoning in the EU (Berny *et al.*, 2007; Berny and Gaillet, 2008; SASA, 2000–2009; Guitart *et al.*, 2010b). The investigation of pesticide poisoning of the Cinereous vulture in Spain revealed that carbofuran, aldicarb and

strychnine account for up to 88% of all cases (Hernández and Margalida, 2008). Between 1998 and 2004, 70 animals of the wild fauna (38 birds and 32 mammals) of northern EL were subjected to analysis and 52.63% of birds and 43.75% of mammals tested positive for toxic substances responsible for their death (Samouris *et al.*, 2007). In EL, it appears that anticholinesterase agents are the main cause of wildlife poisoning followed by sporadic incidents due to warfarin and cyanide salts (Samouris *et al.*, 2007; Guitart *et al.*, 2010b).

## Insecticides

Carbamates were found to be responsible for the majority of acute dog poisoning incidents in BE, CZ and IT (Giuliano Albo and Nebbia, 2004; Modra and Svobodova, 2009; Vandenbroucke *et al.*, 2010). Apart from being responsible for 46.9% of positive cases involving sheep and 66.7% involving goats in northern EL (Guitart *et al.*, 2010a), carbamates were also found to be the cause of a recent case involving the poisoning of 55 members of a dairy goat herd following the consumption of drenched carnations at a nearby greenhouse (Giadinis *et al.*, 2009). Moreover, carbofuran has been, by far, the most common cause of wildlife toxicoses in BE and CZ in the last decade (Modra and Svobodova, 2009; Vandenbroucke *et al.*, 2010).

Organochlorine insecticides such as DDT were banned many years ago due to their persistency in the environment and accumulation in the fatty tissues of organisms. However, failure to meet handling regulations of toxic substances and/or waste materials has recently led to cattle poisoning by DDT in CZ (Modra and Svobodova, 2009).

## Rodenticides

The presence of rodenticides and more specific second-generation anticoagulants was analytically confirmed in the majority of suspected companion animal poisoning cases in IT and to a lesser extent in BE, ES and FR (Berny *et al.*, 2010a). Indeed, a study in FR between 2004 and 2007 showed that dogs were involved in cases of anticoagulant poisoning (over 60%) more than other domestic species (Berny *et al.*, 2010b). Similarly, in CZ and AT, anticoagulant rodenticides were recorded as a significant cause of acute poisoning in dogs after deliberate abuse or misuse (Wang *et al.*, 2007; Modra and Svobodova, 2009). Additionally, anticoagulant rodenticides rank among the rare but nevertheless reportable causes of hemorrhage and epistaxis in cats and dogs (Kohn *et al.*, 2003; Mylonakis *et al.*, 2008). Strychnine, although banned in the majority of EU countries, is still reported



as the most common form of rodenticide toxicity in EL, ES, FR and certain regions of IT (Amorena *et al.*, 2004; Berny *et al.*, 2010a).

Hares and rabbits were also found to be significantly present in cases of anticoagulant exposure and account for almost 50% of the submitted cases in FR, followed by scavengers and predators of poisoned rats (Berny *et al.*, 2010b). In the same country, two polecats and an American mink were also reported poisoned by bromadiolone during a screening process aimed at identifying the reason behind the decline in the endangered European mink population (Fournier-Chambrillon *et al.*, 2004). The Predatory Bird Monitoring Scheme (PBMS) and the WIIS in the UK have also identified anticoagulant rodenticides as a common contaminant of wildlife and a cause of mortality (SASA, 2000–2009; DEFRA, 2007).

### Other pesticides

Metaldehyde is usually found in pellets used for molluscicide treatment and that are palatable and easily digested by animals. Dog and cat poisoning by metaldehyde is a big issue in CZ and the UK and has alarmingly increased in other EU countries such as BE, FR and IT where slug pellets are used extensively, not only in farmland but also in home gardens (Modra and Svobodova, 2009; Berny *et al.*, 2010a). Cases of metaldehyde poisoning involving cattle and pigs having access to stored bags of slug pellets have been recorded in the UK (Sharpe and Livesey, 2005).

Occasionally, the herbicide paraquat has been involved in cases of pet poisoning in EL, ES and IT, whereas glyphosate has had minimal impact on companion animal health in FR (Berny *et al.*, 2010a). Other herbicides and fungicides have been infrequently involved in the poisoning of companion animals in the EU.

## OIL

The two most recent oil spills in the EU occurred on the Atlantic coast: the Erika oil spill in December 1999 (FR), and the Prestige oil spill (ES, FR and PT) in November 2002. The Erika oil spill resulted in over 63,000 birds being stranded on beaches, mostly common guillemots (75%), common scoters (4%) and razorbills (4%). Only 6% of them could be released subsequently. The Prestige oil spill involved heavy fuel oil and the total number of birds affected was estimated to be between 115,000 and 230,000, of which only 23,181 could be collected. It was later demonstrated that the Prestige oil spill had a negative effect on the reproductive ability of the local peregrine falcon in Basque Country (Guitart *et al.*, 2010b).

## FEED ASSOCIATED TOXICANTS

As far as companion animals are concerned, feed-related poisoning incidents reported in the last decade involve the accidental intake of chocolate, grapes, raisins, onions, cannabis products and ethanol by dogs (Kammerer *et al.*, 2001; van Wuijckhuise and Cremers, 2003; Sutton *et al.*, 2009; Berny *et al.*, 2010a; Gunning *et al.*, 2010). Dogs may also be prone to the ingestion of theobromine from garden mulch made of cacao bean shells. Ionophores such as lasalocid have been used as an additive in coccidiostatic feed for poultry. Espino *et al.* (2003) described a case of three hunting dogs in ES that developed acute neurological signs associated with lasalocid poisoning after the consumption of several broilers.

Current trends in feed-related livestock poisoning are related to the accidental presence of industrial chemicals/contaminants or to cross-contamination of feed batches with drugs/additives resulting in the exposure of non-target animals. An example of the former is the Belgian dioxin incident in 1999 in which a tank of recycled fats used to produce animal feeds was accidentally contaminated with approximately 100 liters of polychlorinated biphenyl oil. The incident was discovered when poultry poisoning resembling the classic chick edema disease broke out in several farms that had received contaminated feeds (Vandenbroucke *et al.*, 2010). Another example is a melamine contaminated feed crisis in the pig industry in ES. Between 2003 and 2006, 300 to 400 Iberian piglets developed nephrotoxicosis subsequent to exposure to melamine and derivatives. Morbidity was 40–60% and mortality 20–40% in the total population of post-weaning piglets (González *et al.*, 2009).

The phenomenon of cross-contamination is well known where ionophores such as monensin, lasalocid, narasin and salinomycin are concerned. They are frequently used as feed additives and coccidiostats for cattle, sheep and poultry. Acute monensin toxicosis in equids is well documented. Sharpe and Livesey (2005) reported a total of 37 ionophore poisoning incidents in the UK between 1990 and 2002 involving sheep, turkey and poultry. Ionophore poisoning has also been reported in cattle in NL where salinomycin had been accidentally added to the feed powder (Huyben *et al.*, 2001). A special case of acute tiamulin-salinomycin toxicosis in pigs due to a pharmacokinetic interaction between both compounds was reported in BE (Vandenbroucke *et al.*, 2010).

Other feed-related poisoning incidents observed during the last decade in the EU involve well-known toxicants such as nitrate, nitrite and ammonia. Sharpe and Livesey (2005) reported three nitrate poisoning incidents involving cattle and one involving sheep in the UK between 1990 and 2002. Several cases of acute mortality in pigs in BE have been described in which still,

standing water was identified as the nitrite source (Vandenbroucke *et al.*, 2010). In the case of cattle, there have been occasional incidents of overconsumption of soybean meal causing acute carbohydrate fermentation and excessive ammonia release leading to nervous symptoms or of ingestion of cultivated and wild onions (*Allium* spp.) resulting in hemolytic anemia and acute death (Vandenbroucke *et al.*, 2010).

## DRUGS AND RELATED COMPOUNDS

Among the many different therapeutic classes of human drugs, non-steroidal anti-inflammatory drugs (NSAIDs), benzodiazepines and barbiturates are the most common contributors to animal poisoning (Berny *et al.*, 2010a). While in the USA several studies dealing with the ingestion of the non-benzodiazepine hypnotic drug zolpidem by dogs have been reported, only one case of cat poisoning has been published in PL in the EU (Czopowicz *et al.*, 2010). In addition, the ingestion of calcipotriol ointment (a structural analog of calcitriol) by a dog was reported in the UK (Torley *et al.*, 2002). In the same EU country, ingestion of hydroxycarbamide (hydroxyurea) tablets by a dog was associated with methemoglobinemia (Wray, 2008). Another well-known accidental ingestion of drugs involves the intake of equine de-worming medication by dogs. Collies are especially known for their breed-dependent adverse reaction to ivermectin (Berny *et al.*, 2010a).

Livestock poisoning cases related to human and veterinary drugs has seldom been reported in the EU in the last decade. Cases include the poisoning of pigs by vitamin D<sub>3</sub> in DE due to the erroneous presence of the vitamin in ready-to-use food mixes (Heinritzi *et al.*, 2000), calves by doxycycline in BE after receiving high doses as treatment for respiratory disorders (Vandenbroucke *et al.*, 2010) and sheep and cattle in the UK due to the misuse of unlabeled or out-of-date diazinon products (Sharpe *et al.*, 2006).

## HOUSEHOLD PRODUCTS

Since dogs and cats share our domestic environment, they are highly exposed to household products (e.g., 720 cases in FR in 2003) (Berny *et al.*, 2010a). Among them, hydrocarbons account for nearly 40% of poisoning incidents, especially in the case of cats exposed to domestic fuel, xylene and other common solvents used in paint and paint solvents. Exposure to these substances usually results in very severe cases with a high mortality rate.

Other common household toxicants include detergents and caustics (bleach, strong acids and bases), accounting for almost 40% of the 720 cases. In 2004, 69 calls related to fertilizer poisoning were registered in FR and most of them (65%) resulted in moderately severe cases with digestive disorders. Similarly in IT, bleach and detergents are often involved in the poisoning of both cats and dogs, whereas petroleum distillate poisoning was reported mainly in cats (Amorena *et al.*, 2004; Giuliano Albo and Nebbia, 2004). In IT and ES, ethylene glycol poisoning involving dogs has been recorded (Berny *et al.*, 2010a) and some recent cases involving cats have been analytically confirmed in Catalonia. Long-term exposure and poisoning of a dog by ethylene glycol has also been reported, eventually leading to renal failure (Goicoa *et al.*, 2003).

## TOXINS

Suspected toxin poisoning has been reported in the case of companion animals (phytotoxins and zootoxins), food producing animals (phytotoxins and mycotoxins) and wild birds (botulism and cyanobacteria).

### Plants

Poisoning or suspected poisoning of companion animals as a result of ingestion of plants is a relatively common occurrence and cats appear to be more sensitive and more at risk than dogs, presumably because they tend to chew on plant leaves (Berny *et al.*, 2010a). Often the cases are related to accidental ingestion of ornamental plants rather than wild plants, particularly at certain times of the year. Cats are generally more likely to be poisoned by household/ornamental plants and dogs are more at risk from wild plants (Giuliano Albo and Nebbia, 2004; Berny *et al.*, 2010a).

Liliaceae and Araceae are the two major plant species involved in poisoning of companion animals in FR; oleander (*Nerium oleander*), castor bean (*Ricinus communis*) and Jimson weed (*Datura stramonium*) cases are also frequently observed (Berny *et al.*, 2010a).

In IT, many ornamental plants lead to dog poisoning such as dumbcane (*Dieffenbachia* spp.), ficus (*Ficus benjamina*), pittosporo (*Pittosporum tobira*), poinsettia (*Euphorbia pulcherrima*), rhododendron (*Rhododendrum hirsutum*), tiger lily (*Lilium tigrinum*) and jessamine (*Jasminum officinalis*) (Amorena *et al.*, 2004; Giuliano Albo and Nebbia, 2004; Berny *et al.*, 2010a). In BE and CZ, black locust (*Robinia pseudoacacia*), tansy ragwort (*Senecio jacobaea*) and oleander have been responsible for

horse poisoning outbreaks (Modra and Svobodova, 2009; Berny *et al.*, 2010a; Vandenbroucke *et al.*, 2010). In addition, horse poisoning resulting from European yew tree (*Taxus baccata*) exposure is very common in BE and FR (Berny *et al.*, 2010a; Vandenbroucke *et al.*, 2010).

Although livestock poisoning by toxic plants is frequent, these cases often remain only suspected, unconfirmed by analytical diagnoses. Livestock are commonly poisoned by bracken fern (*Pteridium aquilinum*), European yew tree, oak trees (*Quercus* spp.) and ragwort (*Senecio* spp.) in many EU countries (Guitart *et al.*, 2010a; Vandenbroucke *et al.*, 2010; VPIS, 2010).

## Other toxins

In the EU, a number of different avian species have been involved in outbreaks of botulism and the species most affected by this was aquatic birds (Guitart *et al.*, 2010b). Botulism outbreaks in poultry, sheep and cattle are fairly common too (Sharpe and Livesey, 2005; Modra and Svobodova, 2009). Cyanobacteria toxic blooms are the cause of mass mortality of wild birds in inland water systems worldwide and two recent episodes have been reported in ES in the Doñana National Park (López-Rodas *et al.*, 2008; Guitart *et al.*, 2010b).

Although mycotoxicoses are commonly suspected, published reports of poisoning are scarce. Episodes involving different species like cattle, pigs, poultry and sheep have been recently reported in BE, CZ, ES, FR, IT, PL and the UK (Sharpe and Livesey, 2005; Modra and Svobodova, 2009; Obremsky and Zielonka, 2009; Guitart *et al.*, 2010a; Moyano *et al.*, 2010; Vandenbroucke *et al.*, 2010).

Reported cases due to venomous bites by viper (*Vipera* spp.), common toad (*Bufo bufo*) and pine caterpillar (*Thaumetopoea pityocampa*) mostly involve dogs (Amorena *et al.*, 2004; Giuliano Albo and Nebbia, 2004; Berny *et al.*, 2010; Lervick *et al.*, 2010).

## CONCLUSIONS

European toxicoepidemiological data demonstrate that pesticides are the most common class of toxicants in all species. Poisoning in companion animals is a frequent problem and dogs are the most commonly poisoned species, followed by cats and horses. In food-producing animals information on poisoning is limited and fragmented and occurs predominantly in cattle followed by sheep and goats. A small percentage of cases is also reported for poultry and pigs. Poisoning episodes in wildlife are widespread and present a major

investigational problem for toxicology and forensic laboratories. Knowledge of the common features of animal poisoning is central to any endeavor to reduce mortality and it is therefore essential that coordinated and integrated efforts are made between European countries for sharing information.

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# Chemicals of terrorism

Tina Wismer

## INTRODUCTION

Chemical agents may be chosen by terrorists as they can be dispersed over large areas and can eventually penetrate even the most well-defended positions. These agents can be deployed against specific targets, and depending on the agent used, the effects can be immediate or delayed. Chemical agents can cause incapacitation, disorientation or death. Many of the more commonly used agents can be produced inexpensively and easily stored.

There are four basic types of military agents that can be used for chemical terrorism: choking agents (chlorine, phosgene), blister agents (mustard, Lewisite, phosgene oxime), blood agents (cyanide, hydrogen cyanide) and nerve agents (tabun, sarin, soman, VX). Chemical warfare agents can be delivered by a variety of methods: bomb, spray tanks; rockets; missiles; land mines; and artillery projectiles (USACHPPM, 2001a). Few indicators of a chemical attack may be evident at first. The initial observation of unusual signs and symptoms that correlate with nerve, vesicant, blood or pulmonary agent exposures should raise immediate suspicion of poisoning.

The history of chemical warfare dates back to the beginning of the last century. The first chemical warfare agent of modern times was chlorine. It was used by the German army at Ypres in 1915 against the Allies. Experiencing some success, the Germans then began to mix chlorine with phosgene, or deployed phosgene alone as a weapon. Phosgene, arsenicals, blister agents and mustard gas were estimated to be responsible for approximately 1.3 million casualties during the war,

including at least 90,000 fatalities (Raffle *et al.*, 1994; Bingham *et al.*, 2001; HSDB, 2005). Phosgene accounted for 80% of all gas deaths.

Chemical warfare agents do not need to be lethal to be disruptive. Although the mortality rate was not as high as with phosgene, mustard gas was the number one cause of casualties in WWI (Raffle *et al.*, 1994). Mustard gas caused severe injuries, and due to its persistence, remained a hazard and barrier to troop movements.

Chemical weapons have been used in a few recent civil wars. Phosgene was used by Egyptian bombers against Yemeni royalist forces in the Yemeni civil war in the 1980s (Evison *et al.*, 2002). The Iraqi military used both mustard gas and HCN against the Kurds leading to as many as 3000 deaths (Somani and Babu, 1989). Iraq also used "Yellow rain" (a mixture of mycotoxins, mustard gas and nerve agents) against Iran in 1984. Approximately 100,000 unprotected soldiers suffered severe and long-lasting injuries. The fatality rate was 20% (Drasch *et al.*, 1987).

With the changing political climate around the world, several terrorist groups have used chemical warfare to bring attention to themselves and further their cause. Aum Shinrikyo (Supreme Truth) has used several chemical attacks recently to promote their agenda. In June 1994, they attempted to assassinate three Japanese judges by releasing sarin gas in the community where they lived. Aum Shinrikyo had modified a truck with a special device to release the sarin gas. Seven people, but not the judges, died and 280 were injured. Emboldened by this partial success, on March 20, 1995, Aum Shinrikyo carried out a sarin gas attack on the Tokyo subway. The terrorists used sharpened umbrellas to puncture lunch boxes and bags filled with dilute sarin.

Eleven people died and more than 5500 were injured (Kaplan and Marshall, 1996). On April 20, 1995, Aum Shinrikyo changed their chemical agent and released phosgene gas in a Yokohama train station, causing injuries to 300 people.

Chemical agents also have their problems as agents of terror. They are most effective in confined spaces. Chemical warfare agents dissipate quickly and degrade or adhere to surfaces. Attacks need to be well coordinated for dispersal of the agent and escape from the area of deployment.

Both livestock and pet animals can be exposed to chemical agents during a terrorist attack. Treatment of animals is secondary to treating human casualties. Humane euthanasia may be the only recourse due to financial or logistical concerns.

## CHLORINE GAS

### Background

Chlorine (Agent CL,  $\text{Cl}_2$ ) has been used as a choking or pulmonary agent for military purposes under the name bertholite (Budavari, 2000). It is a greenish-yellow diatomic gas with an irritating, pungent, or suffocating odor (Lewis, 1997; Budavari, 2000; NIOSH, 2007). Chlorine gas is heavier than air and will settle in low areas.

### Pharmacokinetics/toxicokinetics

The initial effects of chlorine gas exposure can appear very rapidly, depending upon the concentration. Due to chlorine's water solubility and chemical reactivity, it can have a greater effect on the lower respiratory tract as a large percentage bypasses the upper airways. Respiratory, dermal and ocular irritation starts immediately and acute lung injury peaks in 12 to 24 hours.

### Mechanism of action

Chlorine gas is a strong irritant, and in concentrated amounts may be corrosive to mucous membranes when inhaled or ingested. When chlorine combines with tissue water it produces hydrochloric acid and reactive oxygen species. These free radicals are potent oxidizers, causing further tissue damage. The damage to the respiratory epithelium leads to alveolar capillary congestion followed by high fibrinogen edematous fluid (Noe, 1963). Hypoxemia results from development of atelectasis,

emphysema and membrane formation. Death usually occurs within 48 hours from cardiac arrest secondary to hypoxia (Decker, 1988).

### Toxicity

Inhalation is the main route of chlorine gas exposure. Chlorine gas is very irritating on contact and can be caustic to the eyes, skin, nose, throat and mucous membranes. Ocular exposure can result in severe or permanent eye injury. Dermal exposure may cause erythema, pain and irritation. Both liquid chlorine and high concentrations of gaseous chlorine can cause dermal burns (Raffle *et al.*, 1994). Signs of exposure to chlorine gas include: rhinorrhea, ataxia, syncope, muscle weakness, dermatitis, dyspnea, tachypnea, pneumonia, bronchospasm and acute lung injury. High concentrations may cause laryngospasm, cardiovascular collapse, tachycardia and respiratory arrest (Noe, 1963). Respiratory symptoms may be immediate or delayed up to several hours depending on the concentration (Bingham *et al.*, 2001). Hypoxia is common and death may be rapid. See Table 8.1 for severity of signs expected after inhalation at certain ppm concentrations of chlorine gas.

Symptoms generally disappear within six hours after mild exposures, but may continue for more than 24 hours with severe exposures. Moderate to severe exposures can result in chronic respiratory dysfunction (Decker, 1988; Schwartz *et al.*, 1990).

Chlorine is both teratogenic and carcinogenic. Chlorine at 100 ppm when given to pregnant rats in their water caused both biochemical and metabolic effects in

TABLE 8.1 Different effects of exposure levels of chlorine gas

Values in ppm	Effect
0.2–3.5	Odor detection (some tolerance develops)
1–3	Mild mucous membrane irritation that can be tolerated for up to 1 h
3	Extremely irritating to the eyes and respiratory tract
5	Severe irritation of eyes, nose, and respiratory tract; intolerable after a few minutes
14–21	Immediate irritation of the throat; dangerous if exposed for 30–60 min
15	Irritation of the throat
30	Moderate irritation of the upper respiratory tract; immediate chest pain, vomiting, dyspnea, cough
35–50	Lethal in 60–90 min
40–60	Toxic pneumonitis and acute lung injury; dangerous for even short periods
430	Lethal over 30 min
1000	Fatal within a few minutes

Lewis (2000), HSDB (2005) and Bingham *et al.* (2001).

the newborns (RTECS, 2006). Carcinogenicity has only been demonstrated in chronic exposures (Morris *et al.*, 1992; RTECS, 2006).

## Treatment

Move animals into fresh air and monitor for respiratory distress. If coughing or dyspnea develops, evaluate for hypoxia, acidosis, respiratory tract irritation, bronchitis or pneumonitis. Oxygen supplementation along with intubation and ventilation may be needed. Beta adrenergic agonists can help if bronchospasm develops (Guloglu *et al.*, 2002).

Flush eyes with copious amounts of room temperature 0.9% saline or water for at least 15 minutes. Fluorescein staining should be performed to check for corneal defects (Grant and Schuman, 1993). Animals should be bathed with copious amounts of soap and water. Chlorine blood concentrations are not clinically useful as it converts directly to hydrochloric acid in the lungs and other tissues.

Animal models have suggested that corticosteroids can hasten recovery from severe chlorine gas poisoning (Traub *et al.*, 2002); however, administration of steroids to exposed humans has not been shown to provide any significant change (Chester *et al.*, 1977). Pigs exposed to chlorine gas responded best to a combination of aerosolized terbutaline and budesonide than to either therapy alone (Wang *et al.*, 2004). Sheep exposed to chlorine gas and then nebulized with 4% sodium bicarbonate had decreased mortality and improved arterial blood gas values (Chisholm *et al.*, 1989).

## Concluding remarks

Rescuers should wear self-contained breathing apparatus (SCBA) and have protective clothing when entering contaminated areas. Chlorine dissipates quickly in warm climates and does not leave an environmental residue (Munro *et al.*, 1999). The potential for secondary contamination is low, as the gas is not carried on contaminated clothing.

# PHOSGENE

## Background

Phosgene (Agent CG, carbonyl chloride,  $\text{CCl}_2\text{O}$ ) is classified as a choking agent. It is a colorless, non-combustible and highly toxic gas. At room temperature phosgene is

easily liquefied (ACGIH, 2005; CHRIS, 2005; Proctor and Hughes, 2004; NIOSH, 2007) and at high concentrations, the gas has an odor described as strong, suffocating and pungent. Lower concentrations are described as smelling like green corn or "haylike" (Raffle *et al.*, 1994; Budavari, 2000; Pohanish, 2002). Phosgene will sink in water (Budavari, 2000; Bingham *et al.*, 2001).

## Pharmacokinetics/toxicokinetics

Dyspnea develops 2 to 6 hours post exposure in most patients, but may be delayed up to 15 hours (Borak and Diller, 2001). With high concentrations ( $>200$  ppm), phosgene can cross the blood-air barrier in the lung and cause hemolysis and coagulopathies (Sciuto *et al.*, 2001).

## Mechanism of action

Phosgene is a lower respiratory tract irritant. Due to its low water solubility and low irritancy of the upper respiratory system, phosgene is able to penetrate deeply into the lungs (Franch and Hatch, 1986). Phosgene gas interacts with water in the lungs, where it is hydrolyzed into hydrochloric acid leading to cellular injury (Murdoch, 1993). Phosgene also acylates sulfhydryl, amine and hydroxyl groups (Borak and Diller, 2001). This results in protein and lipid denaturation, changes in membrane structure and disruption of enzymes. Phosgene increases pulmonary vascular permeability, leading to increased fluid accumulation in the lung interstitium and alveolae. This fluid accumulation results in gas diffusion abnormalities and pulmonary edema (Diller, 1985; Ghio *et al.*, 1991). Phosgene also decreases energy metabolism and disrupts the glutathione redox cycle. Animals exposed to phosgene have elevated levels of leukotrienes and neutrophil chemotactic agents. Neutrophils congregate in the lung releasing cytokines and other reactive mediators which contribute to pulmonary injury (Ghio *et al.*, 1991; Sciuto *et al.*, 1995). Bronchiolar epithelium is damaged, resulting in local emphysema and partial atelectasis. Death is due to anoxia secondary to pulmonary edema.

## Toxicity

Most exposures to phosgene are from inhalation. The odor of phosgene gas is not sufficient to warn individuals of toxic levels and with high concentrations, olfactory fatigue can occur (Borak and Diller, 2001; ACGIH, 2005). The degree of pulmonary injury relates to the concentration and length of exposure (Bingham *et al.*, 2001) and

initial symptoms are not considered to be a good indicator of prognosis (Diller, 1985).

Exposure to concentrations less than 3 ppm may not be immediately accompanied by symptoms, but delayed effects usually occur within 24 h of exposure. Concentrations as low as 3 to 5 ppm can cause immediate conjunctivitis, rhinitis, pharyngitis, bronchitis, lacrimation, blepharospasm and upper respiratory tract irritation and extended (170 minutes) exposure was fatal (Diller, 1985; Wells, 1985; Proctor and Hughes, 2004). A dose of 50 ppm for 5 minutes may cause pulmonary edema and rapid death (Chemstar, 1996; Borak and Diller, 2001; RTECS, 2006).

A lag time of 1 to 6 h before the onset of respiratory distress and pulmonary edema is common with acute, high-dose exposures ( $>50$  ppm/min). Signs can be delayed for up to 24 (most common) or 72 h with exposures to lower concentrations (Proctor and Hughes, 2004; Pohanish, 2002). Thoracic radiographs can show evidence of pulmonary edema within 1 to 2 h of high-dose exposure, 4 to 6 h after moderate exposure and approximately 8 to 24 h after low-dose exposure (Diller, 1985).

Progressive dyspnea, productive cough, cyanosis and hemoptysis are common initial signs following exposure (Wells, 1985; Borak and Diller, 2001). Hypoxemia and hypoventilation are common secondary to respiratory distress (Wells, 1985). Animals may develop secondary GI, hepatic, renal or brain injury, due to lack of oxygenation. In a dog model, severe phosgene poisoning caused initial bradycardia followed by tachycardia and progressive hypotension (Patt *et al.*, 1946). Cardiac failure may occur secondary to severe pulmonary edema.

Direct contact with the liquefied material can cause dermal burns (Proctor and Hughes, 2004) and severe eye irritation, corneal opacification and frostbite (Proctor and Hughes, 2004). Corneal opacification has also been produced in cats exposed to highly concentrated phosgene gas (Grant and Schumann, 1993).

Prognosis is directly related to the extent of pulmonary injury. If the animal survives 24 to 48 hours, the prognosis improves. Pulmonary edema begins to resolve after 2 to 3 days. Survivors may have suppressed natural killer cell activity and are more susceptible to infectious agents. Secondary infections may become evident 3 to 5 days after exposure. They may also have persistent exertional dyspnea, reduced exercise capacity and abnormal pulmonary function tests (Borak and Diller, 2001).

## Treatment

If inhalation exposure occurs, remove animals to fresh air (higher ground) and monitor for respiratory distress. Exposed skin should be washed with soap and water. Exposed eyes should be flushed for 15 minutes with

tepid water. Asymptomatic animals should be monitored for 12–24 hours for development of pulmonary edema (Borak and Diller, 2001). Symptomatic animals should receive 100% oxygen. If arterial blood gases or  $pO_2$  continues to fall, intubation and ventilation is recommended. Plasma phosgene levels are not clinically useful and there is no specific antidotal agent. Management of patients with pulmonary edema from phosgene is the same as for an ARDS (acute respiratory distress syndrome) patient. Diuretics are not indicated and can worsen volume depletion. Mechanical ventilation with oxygen and PEEP (positive end-expiratory pressure) is the mainstay of treatment.

Intravenous fluids can help with cardiovascular support, but monitor closely for signs of volume overload. Colloids are preferred as they will remain in the vascular space for a longer period of time. Most arrhythmias will resolve with adequate oxygenation.

Nebulized beta adrenergic agonists are recommended if bronchospasm occurs. In a rabbit inhalation study, animals exposed to toxic levels of phosgene were dosed with intravenous aminophylline and subcutaneous terbutaline. If given within 10 minutes post exposure, it prevented noncardiogenic pulmonary edema (Kennedy *et al.*, 1989). N-acetylcysteine (Mucomyst<sup>®</sup>) administered intratracheally to rabbits 45 to 60 min after inhalational exposure to phosgene (1500 ppm/min) decreased pulmonary edema, production of leukotrienes, lipid peroxidation and maintained normal glutathione levels as compared to rabbits exposed to phosgene only (Sciuto *et al.*, 1995). Ibuprofen was shown to protect against acute lung injury from phosgene in rats and rabbits (Guo *et al.*, 1990). Supplemental oxygen and sodium bicarbonate were beneficial in dog experiments (Mautone *et al.*, 1985). Aerosolized surfactant improved lung compliance in a dog model, but the effects of the surfactant on the development or resolution of pulmonary edema were not determined (Mautone *et al.*, 1985). Animal experiments also suggest that corticosteroids, prostaglandin E1 and atropine may be helpful in treating phosgene-induced pulmonary edema (Chemstar, 1996).

## Concluding remarks

Phosgene is heavier than air and will pool in low-lying areas. This heavy vapor density, 3.4 times that of air, made phosgene practical for trench warfare. Phosgene is considered to be nonpersistent in the environment. Air concentrations are reduced by atmospheric water, such as rain or fog (Borak and Diller, 2001). Rescuers should wear proper protective clothing when treating exposed patients. Fortunately, the potential for secondary contamination of rescue personnel is low, as the gas does not persist in fabric or leather. Phosgene is used extensively



in industry as a chemical precursor and this widespread availability makes it an attractive agent for terrorist use.

## MUSTARD GAS

### Background

Mustard gas (Agent H,  $C_4H_8Cl_2S$ ) is a vesicant agent used in chemical warfare. It causes necrosis of the skin, eyes and respiratory tract. It is an organic lipophilic sulfide which is a bifunctional alkylating agent (Borak and Sidell, 1992; Lewis, 1997). Mustard agent is a clear oily liquid in its pure state. Due to impurities, however, it is normally amber to black, or yellow to brown in color (Munro *et al.*, 1999; USACHPPM, 2001b; HSDB, 2005). The liquid becomes aerosolized when dispersed by spraying or by explosive blast from a shell or bomb (USACHPPM, 2001a). Mustard gas has an odor resembling garlic, mustard or horseradish. Systemic poisoning occurs more easily in warm climates than in temperate ones. Mustard gas is still considered a major threat by the U.S. military, as it is easily manufactured and is both incapacitating and lethal.

### Pharmacokinetics/toxicokinetics

Mustard gas is toxic by all routes of exposure (oral, inhaled, dermal and ocular) (EPA, 1985a; Sidell *et al.*, 1997; Lewis, 2000; Pohanish, 2002). Skin penetration of both the liquid and vapor is rapid, and mustard causes both localized cellular and systemic damage (NATO, 1973). Mustard penetrates down hair follicles and sweat glands within minutes. Dermal absorption of mustard varies by species. About 20% of a dermal dose is absorbed through human skin, while up to 75% is absorbed through the skin in rats (Hambrook *et al.*, 1992; Smith, 1999). Ocular absorption also happens within minutes. The latent period for absorption is inversely related to the dose, temperature and humidity (NATO, 1973). Lesions develop within 2 to 3 hours with high-level exposures, and 8 to 10 hours after milder exposures (Requena *et al.*, 1988).

The chemical reaction with biological tissue occurs rapidly but symptoms are typically delayed by several hours (Grant and Schuman, 1993; Sidell *et al.*, 1997). Dog studies show that equilibrium between blood and tissues was achieved within 5 minutes after inhalation (IARC, 1975). Once inside the body, mustard accumulates (in descending order) in fat, skin with subcutaneous fatty tissue, brain, kidney, muscle, liver, cerebrospinal fluid, spleen and lung (Drasch *et al.*, 1987; Somani and Babu,

1989). There are no measurable levels of mustard in the liquid from a skin blister. Urine is the major route of excretion in rabbits, mice and rats. After IV administration in rats and mice, the majority is excreted in 72 to 96 hours (Maisonneuve *et al.*, 1993; Dacre and Goldman, 1996). Only about 6% is eliminated in the feces (IARC, 1975). Bone marrow damage is not evident for 3 to 5 days post exposure. Leukopenia usually occurs at day 7 to 10 following exposures (Garigan, 1996).

### Mechanism of action

The mechanism of action for cellular damage by mustard is unknown, but four theories have been proposed: alkylation of deoxyribonucleic acid, oxidative stress upon cell components, depletion of glutathione, and an inflammatory response (Smith *et al.*, 1995). Mustard gas is a bifunctional alkylating agent. It forms covalent cross-links between the double strands of DNA which inhibits DNA synthesis. Mustard-induced blistering appears to be correlated to DNA damage in the basal cells (Andreassi, 1991; Cowan *et al.*, 1998). Mustard penetrates the skin and damages the cells separating the epidermis from the dermis. Mustard disrupts the hemidesmosomes leading to blister formation between the two layers (Sidell *et al.*, 1997). Skin biopsies from mustard gas blisters revealed a separation of the basal cells from one another and the development of multinucleated cells (Bismuth *et al.*, 1995).

Mustard also causes oxidative stress on intracellular molecules. Mustard forms an electrophilic ethylene episulfonium intermediate in the aqueous of the cytosol (Smith, 1999). The episulfonium ion reacts with sulfhydryl groups leading to increased intracellular calcium. The increased calcium level breaks down the microfilaments needed to maintain cell integrity and induces apoptosis by activating endonucleases, proteases and phospholipases (Smith *et al.*, 1995). Mustard gas may also exert its toxicity via depletion of glutathione. Without glutathione, reactive oxygen species react with membrane phospholipids causing loss of membrane function, fluidity and integrity. Inflammation and sulfur mustard-increased proteolytic activity are also implicated in contributing to mustard pathology (Cowan *et al.*, 1998).

### Toxicity

Mustard gas is a radiomimetic as it produces lesions similar to radiation (Andreassi, 1991; Sidell *et al.*, 1997). Tissues with high cell turnover are the most affected. Being a vesicant, mustard gas can produce erythema, severe pruritus, blistering, ulceration and necrosis of

exposed skin (Borak and Sidell, 1992; ITI, 1995; Dacre and Goldman, 1996; Budavari, 2000; Pohanish, 2002). With dermal exposure, the skin initially appears pale and then becomes erythematous within a few hours of exposure (Requena *et al.*, 1988). Blistering usually starts on the second day and progresses for several more days. Erythema disappears in 3–7 days, while the ulcers take 6–8 weeks to heal (Garigan, 1996; Sidell *et al.*, 1997). Brown or black hyperpigmentation usually occurs after resolution of the burns, especially in areas with thinner skin. Skin involvement is most severe at warm and moist sites (genitalia, perineal regions, groin, skin folds and axillae). This is due to the high number of sweat glands in these areas. The smallest reported blister-causing dose on the skin is 0.02 mg of mustard (Smith *et al.*, 1995).

The eyes are very sensitive to the effects of mustard gas (NATO, 1973; Borak and Sidell, 1992; Dacre and Goldman, 1996). Pain, lacrimation, corneal ulceration, along with photophobia, swelling, blepharospasm and blindness can be seen (Garigan, 1996). Conjunctivitis appears early, developing 4 to 6 hours after exposure. Eye lesions have been reported at a Ct (concentration  $\times$  time) of 10 mg-min/m<sup>3</sup> (Sidell *et al.*, 1997). Porcelain-white areas in the episcleral tissues adjacent to the cornea and formation of large, tortuous, sausage-shaped varicose veins are pathognomonic signs of mustard gas poisoning (Grant and Schuman, 1993). Chronic conjunctivitis and keratopathy has been reported in people (Blodi, 1971).

The main non-dermal toxic effects are on the respiratory tract. Irritation or ulceration of the respiratory tract can occur (Vogt *et al.*, 1984; Borak and Sidell, 1992; Dacre and Goldman, 1996; ITI, 1995; Budavari, 2000). Cough, dyspnea and pulmonary edema may occur up to 24 hours after inhalation. One ppm in air is a lethal concentration for dogs and 0.650 ppm mustard results in a 33% mortality rate in rabbits (OHM/TADS, 2005). See Table 8.2 for LD<sub>50</sub> information in various species by multiple routes of exposure. Mild pulmonary exposures produce rhinorrhea, sneezing, epistaxis and cough within 12–24 hours of exposure. Large exposures can cause pulmonary damage. In severe cases, hypoxia and respiratory acidosis are seen. Seizures appear to occur only following extremely high acute doses (Sidell *et al.*, 1997).

Leukopenia, thrombocytopenia, pancytopenia and anemia have all been reported due to depressed myelopoiesis from destruction of precursor cells in the bone marrow (Vogt *et al.*, 1984; Borak and Sidell, 1992; Dacre and Goldman, 1996). Bone marrow aplasia can be seen in severe cases. Secondary to bone marrow damage, overwhelming infection can result in death (Sidell *et al.*, 1997).

Mustard gas is a possible human and animal teratogen. It has been linked to an increased incidence of cleft lip and cleft palate in Iranian children born during

TABLE 8.2 LD<sub>50</sub> for mustard gas

Species	Route	LD <sub>50</sub> (mg/kg)
Human	Oral	0.7
	Dermal	100
Mouse	Dermal	92
	SQ	20
Rat	Oral	17
	Dermal	5
	SQ	1.5

Lewis (2000) and RTECS (2006).

the Iran–Iraq war (Taher, 1992). Abnormalities of the musculoskeletal system were observed in the offspring of rats orally dosed with mustard gas, but such effects may only occur at doses high enough to be toxic to the dams (Dacre and Goldman, 1996; RTECS, 2006). Other rat and rabbit studies showed no correlation (Hackett *et al.*, 1987). Mustard gas is considered both carcinogenic and neoplastic. Mustard gas has caused carcinomas in the skin, appendages, lungs, thorax and blood (leukemia) of rats and mice via both inhalation and IV exposure (RTECS, 2006).

## Treatment

Move animal into fresh air. Do not induce emesis. Activated charcoal administration after oral ingestion is controversial. It appears to have some beneficial effects if administered within one hour of ingestion. Sodium thiosulfate has been used as a “mustard scavenger” and giving 2% sodium thiosulfate solution orally may help in cases with ingestion exposures (Borak and Sidell, 1992; Dacre and Goldman, 1996). If signs or symptoms of esophageal burns are present, consider endoscopy to determine the extent of injury. Perforation and stricture formation could result.

Flush eyes with tepid water for at least 15 minutes. After flushing, instill 2.5% sodium thiosulfate to help neutralize the mustard. Time to decontamination is very important with ocular exposures. Mustard droplets disappear from the eye very quickly, and late flushing of the eye generally provides no benefit (Sidell *et al.*, 1997). Topical antibiotics and mydriatics should be used if corneal lesions are present. Corneal transplants have been performed on some human patients with good results and may be considered for some valuable animals (Blodi, 1971).

Several general dermal decontamination methods can help reduce the toxicity of mustard gas. Bathe animals with copious amounts of soap and water. Dermal decontamination needs to be implemented quickly, as once mustard has reacted with the skin, it cannot be easily removed (Sidell *et al.*, 1997). A 2.5% sodium thiosulfate

solution has been used to neutralize dermal mustard exposures (Garigan, 1996). Dilute (0.5%) hypochlorite solutions may also be used for skin decontamination (Borak and Sidell, 1992). Clays, such as Mineral Cationic Carrier (MCC®: Kodona; Zagreb, Croatia), a synthetic zeolite, have shown efficacy in dermal decontamination with mustard gas exposures (Vucemilovic *et al.*, 2008). Monitor for dermal effects, which are delayed and progressive. Dermal lesions behave like a chemical burn or radiomimetic effect. Topical silver sulfadiazine should be applied to all affected areas. In a mouse model, topically applied dexamethasone and diclofenac reduced inflammatory parameters when applied within 4 hours (Dachir *et al.*, 2004). Healing can take weeks to months and infection is common (Borak and Sidell, 1992). Removal and debridement of closed blisters is controversial. Blisters should be left intact until they rupture spontaneously or unless they are extremely large or inhibit motion (Roberts, 1988). Tetanus toxoid should be given to all equine and ovine patients. Tetanus prophylaxis of other species should be determined on a case-to-case basis.

Monitor for coughing and respiratory distress. If respiratory abnormalities occur, monitor arterial blood gases and/or pulse oximetry, and thoracic radiographs. Thoracic radiographs may show an infiltrate within the first two days (Smith, 1999). Nebulization of 2.5% sodium thiosulfate may help neutralize the mustard gas. N-acetylcysteine (Mucomyst®) is also a potential mustard gas antagonist (Garigan, 1996). A loading dose of 140mg/kg should be given, followed by 40mg/kg every 4 hours for a total of 17 doses (Garigan, 1996). Administer oxygen and inhaled beta agonists if needed. Intubation and ventilation may be needed. Melatonin can be beneficial in acute mustard toxicosis. It is a scavenger of both oxygen- and nitrogen-based free radicals and regulates gene expression of antioxidant enzymes (e.g., SOD, GSH-Px) (Reyes-Toso *et al.*, 2004; Tan *et al.*, 2007). Melatonin also decreases inflammation by blocking pro-inflammatory cytokines and sparing ATP production (Tan *et al.*, 2007). Combinations of parenteral dexamethasone, promethazine, vitamin E and heparin have shown protective effects against mustard gas poisoning in laboratory animals (Vojvodic *et al.*, 1985; Requena *et al.*, 1988).

Monitor CBC with platelets for 2 weeks after exposure. Leukopenia develops at 7 to 10 days following a severe acute exposure (Garigan, 1996). Prophylactic antibiotics are not recommended, but a broad spectrum antibiotic should be started if leukopenia develops (Sidell *et al.*, 1997). Antibiotic choice should be based on culture and sensitivity if possible.

Mustard can be detected in air, urine and body tissues using different methods. Mustard gas or its thiodiglycol metabolite can be detected in urine up to a week after

acute exposure using gas chromatography-mass spectrometry (Vycudilik, 1985). Other than for confirming the diagnosis, measuring mustard gas levels is not likely to be of value in the management of the patient.

## Concluding remarks

Due to its low volatility, mustard is persistent in the environment. It persists for shorter periods of time in a hot climate, but reaches higher vapor concentrations more rapidly. In temperate areas mustard may persist for more than 1 week, but in desert conditions, persistence is reduced to about 1 day. Mustard will bind to vegetation for days to weeks (USACHPPM, 2001b). Detection tubes can be used to monitor airborne levels of mustard gas (IARC, 1975).

The potential for secondary contamination is high. Rescue personnel must wear protective clothing, eye protection and a respirator (HSDB, 2005). Mustard gas penetrates wood, leather, rubber and paints. Medical personnel treating mustard-exposed patients have developed toxicity.

## LEWISITE

### Background

Lewisite ( $C_2H_2AsCl_3$ ) is a substituted arsine. Lewisite was first synthesized in 1918 by a research team headed by U.S. Army Captain W.L. Lewis. It is an oily vesicant (blister-causing) liquid with potential terrorist use. Lewisite smells like geraniums. Pure Lewisite is colorless, but impurities and age cause the color to darken (amber to black or violet to brown, to olive-green) (HSDB, 2005). Lewisite remains a liquid at low temperatures and is persistent in colder climates. It hydrolyzes rapidly, making it difficult to maintain a biologically active concentration on a humid day (Sidell *et al.*, 1997; AAR, 2000; Lewis, 2000; HSDB, 2005). The synthesis of Lewisite involves adding arsenic trichloride to acetylene, and using aluminum chloride as a catalyst. This results in a mixture of about 20% Lewisite, other arsine compounds and an explosive component. Lewisite can be dispersed in air as a very fine droplet spray over a large distance (Grant and Schuman, 1993; Lewis, 2000).

### Pharmacokinetics/toxicokinetics

Lewisite can cause systemic signs when ingested, inhaled or when absorbed dermally or ocularly (Sidell *et al.*, 1997; HSDB, 2005). Inhalation of vapor causes

immediate pain and if high enough concentrations are inhaled, death can be seen within 10 minutes (DeRosa *et al.*, 2002). Dermal absorption occurs within 3 to 5 minutes, especially following liquid exposures (Sidell *et al.*, 1997). The volume of distribution of arsenic after Lewisite administration is several liters/kilogram, indicating extensive tissue distribution (HSDB, 2005). A distribution study using rabbits revealed that the liver, lungs and kidneys had the highest concentration of arsenic after Lewisite administration (greater than seven times blood concentration). Arsenic can cross the placenta and is passed into the milk, and nursing animals may be at risk (Barlow and Sullivan, 1982).

Animal studies showed that excretion of Lewisite oxidation products into the bile caused focal necrosis of the liver and necrosis of biliary vessel mucosa with peribiliary hemorrhages (Munro *et al.*, 1999). The same study in rabbits found that arsenic was eliminated with a half-life in blood of 55 to 75 hours (HSDB, 2005). The excretion of oxidized Lewisite products into the bile by the liver may result in injury to the intestinal mucosa (Munro *et al.*, 1999).

## Mechanism of action

Besides being a vesicant, Lewisite is an arsenical compound which causes systemic effects. Lewisite directly effects enzyme systems. The exact mechanism of action is unknown, but it inhibits a variety of enzymes (pyruvic oxidase, alcohol dehydrogenase, succinic oxidase, hexokinase and succinic dehydrogenase) (DeRosa *et al.*, 2002). Lewisite binds with thiol groups on these enzymes, resulting in decreased ATP production. Ocular injuries following Lewisite exposure are due in part to the liberation of hydrochloric acid. Deep penetration of Lewisite into the cornea and aqueous humor causes rapid necrosis (Goldfrank *et al.*, 1998).

Lewisite causes increased capillary permeability. Systemic absorption and increased permeability can cause a significant loss of blood plasma volume and is called "Lewisite shock" since it is similar to that of shock observed in severe burns. The leakage of fluid into the extravascular space results in hypotension (Sidell *et al.*, 1997). The exact mechanism of increased capillary permeability is not known. Theories include a capillary dilating material released from skin or tissue, or alternatively, enhanced permeability from an interference with the metabolism of capillary endothelial cells (Goldman and Dacre, 1989). Lung capillaries appear to be the most affected due to absorption via the respiratory tract and first pass through the lungs following dermal exposure. Pulmonary edema or acute respiratory distress syndrome can develop (Sidell *et al.*, 1997).

TABLE 8.3 LD<sub>50</sub> for Lewisite

Species	Route	LD <sub>50</sub> (mg/kg)
Mouse	Dermal	12
Rat	Dermal	15
	Subcutaneous	1
	Oral	50
Human	Dermal	30

RTECS (2006), Sidell *et al.* (1997) and DeRosa *et al.* (2002).

## Toxicity

Due to its method of dispersal (bursting charge of explosive), the main routes of absorption are dermal and respiratory. Lewisite first acts as a vesicant, then as a pulmonary irritant and finally as a systemic poison. Lewisite is similar to mustard gas in that it damages the skin, eyes and airways; however, it differs in that its clinical effects appear within seconds of exposure and it is about 10 times more volatile than mustard gas (Budavari, 2000). Exposure to Lewisite is very painful, in contrast to mustard. See Table 8.3 for LD<sub>50s</sub> of various species by different routes of exposure.

Dermal contact results in immediate pain. Both vapor and liquid Lewisite can penetrate skin. Reddening of the skin becomes evident within 15 to 30 minutes after exposure (EPA, 1985a; Sidell *et al.*, 1997; Pohanish, 2002). Evidence of tissue destruction (grayish epithelium) will be present within minutes of skin contact (Goldman and Dacre, 1989; Sidell *et al.*, 1997). Severe blisters develop within 12 hours after exposure. The blisters may rupture, usually about 48 hours after occurrence, with copious amounts of fluid seeping from the site. With dermal exposure, as little as 0.5 mL may cause severe systemic effects, and 2 mL may be lethal. Severe edema can be seen due to the Lewisite's increased capillary permeability. Dermal burns are generally deeper than those with mustard gas. Healing occurs much faster than with sulfur mustard-induced lesions and is generally complete within 4 weeks.

Ocular contact causes immediate pain, lacrimation and blepharospasm. Permanent blindness may occur if eye exposure occurs for more than one minute without rapid decontamination (EPA, 1985a; Pohanish 2002). A small droplet (1 µl) can cause perforation and loss of vision (Sidell *et al.*, 1997).

Inhalation of the Lewisite vapor may result in irritation to nasal passages, profuse nasal discharge and violent sneezing (HSDB, 2005). Inhalation of 6 ppm can be lethal (USACHPPM, 2001c). Following inhalation of vapor, coughing and hemoptysis commonly occurs (Sidell *et al.*, 1997; HSDB, 2005). Lesions following Lewisite inhalation are similar to the lesions produced by mustard gas exposures. Dogs that inhaled lethal doses died of necrotizing pseudomembranous



laryngotracheobronchitis (Goldman and Dacre, 1989). A thick membrane was noted in the nostrils, larynx and trachea with purulent bronchitis. Edema, hemorrhage and emphysema were seen in the lungs (Goldman and Dacre, 1989). Death can occur within 10 minutes with high concentrations (EPA, 1985a).

Unlike arsenic or mustard gas, Lewisite does NOT cause damage to the bone marrow or immunosuppression (Sidell *et al.*, 1997). Even though Lewisite is a substituted arsine, it also does not appear to directly cause hemolysis of the red blood cells (HSDB, 2005). Hypovolemia, secondary to fluid loss, can be severe enough to cause renal dysfunction. Arrhythmias may occur as a result of hypovolemia rather than a direct toxic effect of Lewisite on the myocardium. Lewisite was fetotoxic to rats and rabbits, but not teratogenic (Goldman and Dacre, 1989; RTECS, 2006). It is a suspected carcinogen due to its arsenic content. Lewisite blood levels are not clinically useful, but an arsenic blood level below 7 µg/100 ml is considered normal.

## Treatment

Move animals to fresh air and monitor for coughing and respiratory distress. Monitor blood gases and SpO<sub>2</sub> in patients with significant exposures. If coughing or difficulty breathing develops, administer oxygen and assist ventilation as needed. Bronchospasm should be treated with inhaled beta agonists and possibly corticosteroids. Monitor electrolytes and PCV as animals can become hemoconcentrated. Crystalloids should be given with caution not to overhydrate the patient (Goldfrank *et al.*, 1998). Consider urinary alkalinization and maintain good urine output. Monitor for liver and kidney failure and secondary infection.

Emesis is not recommended due to the irritant and vesicant nature of Lewisite. Dilute oral ingestions with milk or water. Activated charcoal is of unknown benefit in Lewisite ingestion and as severe irritation or vesication (blistering) of the esophagus or gastrointestinal tract is likely to occur it is not recommended. Endoscopy may be used to determine the extent of injury. Perforation and stricture formation may occur after ingestion.

Flush eyes with copious amounts of tepid water for at least 15 minutes. A 5% BAL (dimercaprol, British Anti-Lewisite) compounded ophthalmic ointment applied within 2 minutes may prevent a significant reaction. Treatment at 30 minutes will lessen the ocular reaction but does not prevent permanent damage (Goldfrank *et al.*, 1998).

Animals should be rinsed with copious amounts of water. A 5% solution of sodium hypochlorite (diluted liquid household bleach) should be used as soon as possible on contaminated skin. Topical application of a 5%

BAL ointment within 15 minutes of an exposure has been reported to be effective in diminishing the blistering effects of Lewisite (Smith, 1999). Wash BAL ointment off after 5 minutes. The ointment may cause stinging, itching or urticaria. Burns should be managed as discussed previously under mustard gas. Pain control is very important.

Chelation is indicated if there is coughing, dyspnea, pulmonary edema or skin burns larger than palm size (Goldfrank *et al.*, 1998). BAL is an effective arsenic chelator, but requires painful deep intramuscular injections and has numerous side effects (hypertension, tachycardia, vomiting, lacrimation, sweating). BAL will increase the clearance rate of arsenic, but it is contraindicated in animals with liver damage. 2,3-Dimercaptosuccinic acid (DMSA, Succimer<sup>®</sup>) appears to be a very effective arsenic chelator in animals (Graziano *et al.*, 1978). DMSA is an oral agent and is relatively non-toxic. It may be used following BAL. 2,3-Dimercapto-1-propanesulfonic acid (DMPS) is related to DMSA and is used for heavy metal poisoning, especially in Europe. It has been effective in protecting rabbits from the lethal effects of Lewisite (Aposhian *et al.*, 1982).

Urine arsenic levels may be tested using several methods. Urinary arsenic levels of <100 µg are considered normal (Proctor and Hughes, 2004). Concentrations between 0.7 and 1.0 mg/L indicate a potentially harmful exposure.

## Concluding remarks

Lewisite can remain in the environment for about 1 day. It reacts with water to yield a solid arsenoxide that also has vesicant properties. When in contact with strong alkalis, Lewisite is decomposed to less harmful substances. The potential for secondary contamination is high and material spilled on clothing may be transferred to rescuers or medical personnel. Lewisite is considered a terrorist threat as it is easy to produce and has a quick onset of signs. Carcasses should be buried deeply (away from water supplies), rendered or incinerated to insure safety of the food supply.

## PHOSGENE OXIME

### Background

Phosgene oxime (Agent CX, "Nettle Rush," CHCl<sub>2</sub>NO) is a halogenated oxime used as a blistering agent in chemical warfare. Other halogenated oximes include diiodoformoxime, dibromoformoxime and monochloroformoxime, but phosgene oxime is the most irritant of the group

and the only one considered a terrorist warfare threat. Phosgene oxime can be found as a liquid or as a colorless, low-melting point crystalline solid, readily soluble in water. The solid form can produce enough vapor to cause symptoms. Phosgene oxime has an unpleasant, peppery and irritating odor. Phosgene oxime is not a true vesicant as it does not cause skin blisters but it does have a rapid dermal corrosive effect. It can be dispersed as a liquid or vapor causing almost immediate tissue damage upon contact.

### Pharmacokinetics/toxicokinetics

Absorption in both dermal and inhalational exposures is complete and rapid (within seconds) (Sidell *et al.*, 1997). Dermal lesions form within seconds. Phosgene will dissolve in sweat and move to other nonexposed areas of the body as it is soluble in water (DeRosa *et al.*, 2002). Pulmonary edema can be evident on thoracic radiographs within 2 hours of high-dose exposure, 4 to 6 hours of moderate exposure and approximately 8 to 24 hours after low-dose exposure (Sidell *et al.*, 1997).

### Mechanism of action

The exact mechanism action is unknown, but it has been proposed that phosgene oxime reacts with SH and NH<sub>2</sub> groups (U.S. Army, 1996; Sidell *et al.*, 1997). Phosgene oxime exerts its greatest effects in the first capillary bed it encounters.

### Toxicity

Both liquid and vaporous phosgene oxime cause intense, immediate pain and local tissue destruction on contact with skin, eyes and mucous membranes (Sidell *et al.*, 1997). Damage to the eyes, skin and airways is similar to that caused by mustard gas. Following dermal contact with either the liquid or vapor, grayish tissue damage may be seen within several minutes. The damaged areas are erythematous and extremely painful. Within one hour, the area becomes edematous. Browning of the skin and blistering occurs the next day. In about 3 weeks, desquamation, necrosis, crust formation and purulent exudate occur. Pain can last for several days. Skin irritation begins at 0.2 mg/min/m<sup>3</sup> (12 seconds) for humans and is intolerable 3 mg/min/m<sup>3</sup> (1 minute) (USACHPPM, 2001d).

Phosgene oxime is very irritating to the eyes. Very low concentrations can cause lacrimation, inflammation and temporary blindness, and high concentrations can cause

permanent corneal lesions and blindness (U.S. Army, 1996; Sidell *et al.*, 1997; USACHPPM, 2001d). Inhalation or oral absorption may cause respiratory tract irritation, dyspnea and pulmonary edema. The non-cardiogenic pulmonary edema may occur after a several hour delay. Death is due to respiratory arrest.

### Treatment

There is no antidote for phosgene oxime exposure. Move animals into fresh air. Emesis is not recommended after oral ingestion because of the irritant and corrosive effects of phosgene oxime. Immediately dilute oral ingestions with milk or water. Activated charcoal is also not recommended after ingestion, since the primary toxicity is expected to be a local corrosive injury rather than systemic effects from absorption. Charcoal may also obscure endoscopy findings and induce emesis.

Flush eyes with tepid water until pH returns to neutrality and remains so for 30 minutes after irrigation is discontinued (Brodovsky *et al.*, 2000). Decontamination after ocular exposure is critical since phosgene oxime is absorbed within seconds. Corneal ulcers should be treated with mydriatic cycloplegics to prevent synechiae development (Grant and Schuman, 1993; Brodovsky *et al.*, 2000). For more severe corneal lesions, topical steroids, citrate, ascorbate and tetracycline or doxycycline may be used to aid in re-epithelialization.

The skin should be flushed with large volumes of water and mild soap. As phosgene oxime reacts so quickly with tissue, decontamination is not expected to be entirely effective after pain has been produced. Chloramine and phenol towelettes are ineffective for dermal decontamination. Isotonic sodium bicarbonate or 0.5% hypochlorite may remove phosgene oxime that has not yet reacted with tissue. Ulcerated skin lesions should be treated just like a thermal burn. Topical silver sulfadiazine is recommended (Roberts, 1988). Healing of dermal lesions can take from 1 month to over a year (U.S. Army, 1996; Sidell *et al.*, 1997). Both sheep and horses should receive tetanus prophylaxis. Other species should be vaccinated at the veterinarian's discretion. Large amounts of opioid analgesics may be needed to help control pain.

Monitor arterial blood gases, pulse oximetry and thoracic radiographs in patients following significant exposures. Non-cardiogenic pulmonary edema may take 12–24 hours to develop. If dyspnea develops, administer 100% humidified oxygen, perform endotracheal intubation and provide assisted ventilation as required. Beta adrenergic agonists may help if bronchospasm develops. Administer IV fluids but ensure that the animal does not become overhydrated (Hoffman, 2002).

## Concluding remarks

Phosgene oxime is considered nonpersistent in the environment. It hydrolyzes rapidly in aqueous alkaline solutions. The potential for secondary contamination is high. Veterinary personnel should wear aprons, rubber gloves and masks when treating decontaminated patients to avoid self-contamination. Phosgene oxime is of interest to terrorists as it penetrates garments and rubber much more quickly than other chemical warfare agents. Phosgene oxime can also be mixed with other chemical warfare agents (e.g., VX). The phosgene oxime will cause skin damage which will increase the dermal absorption of the second agent.

## CYANIDE AND HYDROGEN CYANIDE

### Background

Cyanogen and cyanogen halides (cyanogen bromide, cyanogen chloride, cyanogen iodide) have been used historically as military chemical warfare agents (Barr, 1985; ACGIH, 2005). Today cyanide is most likely to be used for a terrorist weapon in the form of hydrogen cyanide or cyanogen chloride.

Hydrogen cyanide (Agent AC, HCN, prussic acid) is a colorless gas with a faint bitter almond-like odor (ACGIH, 2005). Hydrocyanic acid is the liquefied form of hydrogen cyanide (Lewis, 1997). Cyanogen chloride (Agent CK, ClCN) is either a colorless irritant gas or liquid with a pungent odor. It was developed to be slightly heavier than air and to have greater environmental persistence. It can release hydrogen chloride and hydrogen cyanide when it contacts water, acids or by thermal decomposition. The water soluble salt forms (calcium cyanide, sodium cyanide and potassium cyanide) will form HCN gas when mixed with a strong acid.

Animals with cyanide poisoning may have an odor of bitter almonds in their gastric or ruminal contents or expired breath. The ability to smell the bitter almond-like odor of cyanide is genetically determined, and 20 to 60% of the population cannot detect its presence (Hall and Rumack, 1986). Cyanide and related compounds are classified as blood agents.

### Pharmacokinetics/toxicokinetics

Cyanide and hydrogen cyanide can be absorbed by inhalation, ingestion, ocularly and through intact skin (Ballantyne, 1983; Hall and Rumack, 1986). Cyanide rapidly diffuses into tissues and irreversibly binds to its target sites. Dermal absorption of significant amounts of

hydrogen cyanide gas has not been reported. There have been no reports of systemic poisoning in humans ocularly exposed to cyanide; however, rabbits have died following ocular exposure to NaCN, KCN and HCN.

Cyanide is distributed to all organs and tissues. The concentration of cyanide in red cells is greater than that in plasma by a factor of 2 or 3 (HSDB, 2005). Cyanide accumulates in neural tissue. It preferentially accumulates in the hypothalamus, with levels about 40% higher compared to the hippocampus, cerebellum and cortex (Borowitz *et al.*, 1994). In acute cyanide intoxication, there are no specific pathologic changes.

Cyanide is metabolized by rhodanase in the liver to thiocyanate (Hall and Rumack, 1986). This reaction complexes cyanide with endogenous sulfur or sulfur supplied from the sodium thiosulfate antidote. Once thiocyanate is formed it is excreted mainly in the urine. Half-life for the metabolism of cyanide to thiocyanate is 20 minutes to 1 hour (Feldstein and Klendshoj, 1954). In animals, the dose of cyanide that produces signs is very close to the lethal dose and death can occur within seconds to minutes.

### Mechanism of action

Cyanide causes its toxicity by forming a stable complex with ferric iron ( $\text{Fe}^{3+}$ ) in cytochrome oxidase enzymes. Since oxygen is unable to re-oxidize the reduced cytochrome a3, this inhibits cellular respiration, oxygen utilization and ATP production, resulting in deprivation of oxygen to the body at the cellular level (Way *et al.*, 1988). In the brain cyanide decreases oxidative metabolism, increases glycolysis and inhibits brain glutamic acid decarboxylase, thereby decreasing GABA (Bingham *et al.*, 2001). The corpus callosum, hippocampus, corpora striata and substantia nigra are commonly damaged in cyanide poisoning (Grandas *et al.*, 1989).

Early in cyanide toxicosis, CNS, respiratory and myocardial depression also contribute to decreased oxygenation of the blood and decreased cardiac output (Hall and Rumack, 1986). There is also evidence of lipid peroxidation by measurement of elevated levels of conjugated dienes in mouse brain and kidneys at 15 and 30 minutes after cyanide exposure (Ardelt *et al.*, 1994). Cyanide salts are irritating upon ingestion and can cause corrosion of the oral, esophageal or gastric mucosa (HSDB, 2005).

### Toxicity

Signs following acute cyanide exposure include syncope, or CNS stimulation, dizziness, dyspnea, seizures, paralysis, apnea and coma (Vogel *et al.*, 1981; Hall and Rumack, 1986). Tachypnea and hyperpnea are followed rapidly by

TABLE 8.4 LD<sub>50</sub> for hydrogen cyanide

Species	Route	LD <sub>50</sub> (μg/kg)
Mouse	IM	2700
	IP	2990
	Oral	3700
Rat	SQ	3700

ACGIH (2005), Bingham *et al.* (2001), Budavari (2000), HSDB (2005), ITI (1995), Lewis (2000), OHM/TADS (2005) and RTECS (2006).

respiratory depression. Signs of severe hypoxia without cyanosis can suggest the diagnosis. Signs in birds are similar to those in mammals. Chickens will pant, have rapid eye blinking, excess salivation and lethargy (Wiemeyer *et al.*, 1986). Mydriasis is common in severe poisonings and blindness may occur from cyanide-induced damage to optic nerves and retina (Grant and Schuman, 1993; Vogel *et al.*, 1981). Nausea, vomiting and abdominal pain may occur, especially after ingestion of cyanide salts (Vogel *et al.*, 1981; Hall and Rumack, 1986; Singh *et al.*, 1989). Metabolic acidosis and lactic acidosis are frequent metabolic derangements. Blood gases show a decreased arterial-venous oxygen saturation difference due to the cellular inability to extract oxygen (Paulet, 1955; Graham *et al.*, 1977).

The blood, both arterial and venous, becomes cherry red from accumulated oxyhemoglobin (Lewis, 2000; Bingham *et al.*, 2001). The skin may also be a bright pink color from the high concentration of oxyhemoglobin in the venous return (HSDB, 2005). On fundoscopic exam, retinal arteries and veins will appear equally red. A reduced arterio-central venous measured oxygen saturation difference may be seen due to the same cellular inability to extract oxygen. This arterialization of venous blood gases occurs early in the process of cyanide poisoning. Tachycardia and hypertension may be seen in the initial phases of cyanide poisoning followed by bradycardia and hypotension in the late phases. Cyanide exposure can produce death within minutes. See Table 8.4 for LD<sub>50</sub>s of HCN by various routes.

Cyanide can be measured in blood. No symptoms are expected at concentrations less than 0.2mg/L, tachycardia can be seen at 0.5–1.0mg/L, obtundation at 1.0–2.5mg/L, coma and respiratory depression at levels greater than 2.5mg/L and death with blood levels greater than 3mg/L (Graham *et al.*, 1977). No adverse reproductive studies were found for cyanide or hydrogen cyanide, but in laboratory animals, cyanide compounds did cause resorptions, malformations and teratogenic effects in offspring (Willhite, 1983).

## Treatment

Move animals to fresh air. Emesis is not recommended due to the rapid progression of the clinical signs and

potential for seizures, coma or apnea. Activated charcoal may be beneficial if administered immediately after ingestion as the absorption of cyanide is rapid. Flush eyes for at least 15 to 20 minutes with tepid water. Wash all contaminated animals thoroughly with soap and water.

Monitor arterial blood gases and serum electrolytes. Administer 100% humidified oxygen with assisted ventilation if needed to maintain an elevated pO<sub>2</sub>. Hyperbaric oxygen therapy is approved for cyanide poisoning by the Undersea Medical Society (Myers and Schnitzer, 1984). It has been suggested to improve clinical outcome, but experimental animal studies have been questionable (Way *et al.*, 1972). For severe acidosis (pH < 7.1) administer sodium bicarbonate, but acidosis may be difficult to correct prior to administration of antidotes in cyanide toxicosis (Hall and Rumack, 1986). Control seizures with benzodiazepines or barbiturates.

Cyanide toxicosis usually occurs and progresses so rapidly that treatment is rarely administered soon enough to be effective. Antidotal agents should be used if the animal is in respiratory distress or a coma. There are several different antidotal agents that can be used. Hydroxocobalamin, a vitamin B<sub>12</sub> precursor, is a cobalt containing chelator. Hydroxocobalamin reverses cyanide toxicosis by combining with cyanide to form cyanocobalamin (vitamin B<sub>12</sub>) (Hall and Rumack, 1987). It has been shown to be effective in treating cyanide-poisoned laboratory animals and has the advantage of producing neither methemoglobinemia nor hypotension, as sodium nitrite does.

Another choice for treatment of cyanide poisoning utilizes several steps. Sodium nitrite is given IV over 15–20 minutes, as soon as vascular access is established. Quick administration can cause hypotension. Sodium nitrite reacts with hemoglobin in the red blood cells forming methemoglobin. The methemoglobin will combine with free cyanide to form cyanomethemoglobin. If possible, monitor methemoglobin levels during nitrite administration. The goal is to maintain methemoglobin levels below 30% (Hall and Rumack, 1986). Follow sodium nitrite with IV administration of sodium thiosulfate. Sodium thiosulfate supplies sulfur for the rhodanase reaction. Thiocyanate is formed and excreted in the urine. Oxygen, combined with traditional nitrite/thiosulfate therapy, provides better results than thiosulfate alone (Way *et al.*, 1972). It is believed that oxygen may reverse the cyanide-cytochrome oxidase complex and aid in the conversion to thiocyanate following thiosulfate administration.

Dicobalt-EDTA (Kelocyanor®) is also highly effective in chelating cyanide. It is used clinically in Europe, Israel and Australia, but it is not available in the United States (Hillman *et al.*, 1974). Another methemoglobin-inducing agent used in some European countries is 4-dimethylaminophenol hydrochloride (4-DMAP). It has a more



rapid onset of methemoglobin production than sodium nitrite. Methemoglobin peaks at 5 minutes after 4-DMAP versus 30 minutes after sodium nitrite. 4-DMAP is coadministered with thiosulfate. Excessive methemoglobin production can be a major complication and hemolysis may occur with therapeutic doses (van Dijk *et al.*, 1986).

Animal studies to identify alternate cyanide antidotes have tested stroma-free methemoglobin solutions, alpha-ketoglutaric acid, chlorpromazine, hydroxylamine, phenoxybenzamine, centrophenoxine, naloxone hydrochloride, etomidate, para-aminopropiophenone and calcium-ion channel blockers (Ashton *et al.*, 1980; Amery *et al.*, 1981; Dubinsky *et al.*, 1984; Leung *et al.*, 1984; Ten Eyck *et al.*, 1985; Johnson *et al.*, 1986; Bright and Marrs, 1987; Yamamoto, 1990; Budavari, 2000). These antidotes have shown some promise in the laboratory setting, but have not been tried during actual poisoning situations.

Blood cyanide levels can be useful in confirming the diagnosis. However, unless the results are available within a reasonable time, it is not clinically useful. Cyanide can be measured by several methods but most take several hours to complete. Biological specimens can be tested using spectroscopy, gas chromatography, gas-liquid chromatography, paper chromatography, ion-specific electrode, fluorimetric, microdistillation and paper strip (Groff *et al.*, 1985; Fligner *et al.*, 1992; HSDB, 2005). Cyanide and thiocyanate levels can be measured in timed urine collections to provide information on cyanide clearance, but it is rarely done clinically. Handling of samples is very important due to the volatile nature of HCN. The loss of cyanide from specimens can be minimized by rapid analysis after death, collection of blood from a closed source, storage in a fluoride-oxalate tube with minimal dead space and frozen at minus 20°C (Bright *et al.*, 1990).

## Concluding remarks

Because HCN is lighter than air, it has a long half-life in air. However, HCN rapidly disperses and is diluted to non-toxic concentrations. Cyanide does not concentrate in soil or plant material, but can mix with water. Water converts HCN gas to HCN liquid. Treat contaminated water with ozone, hydrogen peroxide or calcium or sodium hypochlorite bleach. The potential for secondary contamination of rescue personnel is high. Boots, gloves, goggles, full protective clothing and a self-contained positive pressure breathing apparatus are needed (AAR, 2000).

As a chemical warfare agent, cyanide is not easy to disseminate; however, it is widely available which increases the chances of its use in terrorist activities (Burklow *et al.*, 2003). Cyanide works much better as a terrorist weapon in an enclosed space.

## MILITARY NERVE AGENTS

### Background

Military nerve agents are probably the most poisonous of the known chemical warfare agents and are sufficiently toxic that even a brief exposure may be fatal. They were originally synthesized by the Germans during WWII in search of alternatives to the embargoes against insecticidal nicotine. Military nerve agents are rapidly acting, anticholinesterase organophosphate (OP) compounds, and are more potent than organophosphate insecticides. Military nerve agents contain a C-P bond that is unique and very resistant to hydrolysis, except in highly alkaline solutions. At ambient temperatures, nerve agents are viscous liquids, not gases.

Military nerve agents are generally divided into "G" agents and "V" agents. The "G" agents (tabun, sarin, soman) were developed during WWII and are called "G" agents because they were first synthesized in Germany. The "G" agents are very volatile and present a vapor hazard. The vapors are more dense than air, thus they stay close to the ground (Garigan, 1996). Tabun (Agent GA,  $C_5H_{11}N_2O_2P$ ) was the first of the "G" agents to be synthesized in 1936, followed by sarin (GB,  $C_4H_{10}FO_2P$ ) in 1938 and soman (GD,  $C_7H_{16}FO_2P$ ) in 1944 (Sidell *et al.*, 1997).

Tabun is the easiest of the "G" agents to manufacture. It is a fruity-smelling (like bitter almonds) combustible colorless to brownish liquid. Contact with bleaching powder generates cyanogen chloride (EPA, 1985c). It may also undergo hydrolysis in the presence of acids or water, releasing hydrogen cyanide (Munro *et al.*, 1999; Budavari, 2000; HSDB, 2005). Sarin is a colorless liquid with almost no odor in its pure state (Budavari, 2000). Soman is a colorless liquid with a fruity or camphor odor. Soman can release hydrogen fluoride when in contact with acids.

The "V" agents ("V" for venomous) were developed in 1954 in the United Kingdom and are more stable than the "G" agents (Sidell *et al.*, 1997). "V" agents, such as VX, contain a sulfur group and are alkylphosphonothiolates; they are more toxic and persistent on surfaces than G-series agents. VX ( $C_{11}H_{26}NO_2PS$ ) is a nonvolatile, amber colored, odorless liquid.

### Pharmacokinetics/Toxicokinetics

Nerve agents can be absorbed following ocular exposure, oral ingestion, inhalation and dermal contact (HSDB, 2005; RTECS, 2006). These nerve agents are absorbed without producing any irritation or other sensation on the part of the exposed person or animal. Inhalation of military nerve agents will have initial effects on the airways within seconds. Inhalation of a large amount of the vapor will result in sudden loss of

consciousness, apnea, flaccid paralysis and seizures within seconds to 2–3 minutes (Sidell *et al.*, 1997). Peak effects are seen within 20–30 minutes and death is usually due to respiratory failure (Berkenstadt *et al.*, 1991). Dermal exposures to nerve agents have a slower onset of action. Exposure to a large drop or more will result in clinical effects within 30 minutes but with small drops a delay of up to 18 hours can be seen. With ingestion, initial symptoms begin in 20 to 30 minutes and are usually gastrointestinal. There is no taste to solutions containing nerve gas agents (Grob, 1956).

Distribution in the body is slightly different for each of the nerve agents. Distribution of sarin is to the brain, liver, kidney and plasma of mice (Little *et al.*, 1986). Radiolabeled soman was evenly distributed throughout the mouse brain after IV administration, with higher levels in the hypothalamus (Wolthuis *et al.*, 1986). Tabun was also found in high concentrations in the hypothalamus after IV administration in mice (Hoskins *et al.*, 1986). An unusual feature of soman toxicity is its apparent storage in body “depots” and release over time. This results in eventual death in animals who survive the initial dose of soman (Wolthuis *et al.*, 1986).

The military nerve agents differ from other OPs in the rapidity of “aging” of the OP-enzyme complex. “Aging” is thought to be due to the loss of an alkyl group, whereby the inhibitor–enzyme complex becomes resistant to reactivation (Young *et al.*, 1999). The half-life ( $T_{1/2}$ ) of aging for soman is within minutes, for sarin is about 5 hours and for both tabun and VX is greater than 40 hours (Garigan, 1996).

The nerve agents are hydrolyzed by plasma and tissue enzymes to their corresponding phosphoric and phosphonic acids. Oxidative enzymes are also involved in metabolism (HSDB, 2005). Sarin is hydrolyzed in the body to isopropyl-methylphosphonic acid (IMPA). IMPA in mice studies was generally present at 20-fold higher concentrations than sarin in most tissues; exceeding sarin by four times in the brain (Little *et al.*, 1986). In mice studies, the majority of administered radioactive sarin was detoxified and excreted by the kidneys (Little *et al.*, 1986). Mouse studies reveal that approximately 50% of injected soman is converted to free pinacolyl-methylphosphonic acid within 1 minute, and the half-life of this metabolite is less than 1 hour (Reynolds *et al.*, 1985). Soman is mainly eliminated via enzymatic hydrolysis, in competition with binding to target acetylcholinesterase (HSDB, 2005).

## Mechanism of action

Organophosphates (OPs) competitively inhibit acetylcholinesterase (AChE) by binding irreversibly to its esteric site (phosphorylation). Inhibition of the AChE

enzyme results in accumulation of acetylcholine and excessive stimulation at muscarinic, nicotinic and CNS cholinergic sites. Increased acetylcholine at autonomic neuro-effector junctions results in increased smooth muscle contractions and secretions, but its effect at skeletal muscle junctions is initially stimulatory (fasciculations), followed by inhibitory (muscle weakness, paralysis). The effects on the sino-atrial node of the heart is inhibitory, causing bradycardia (Namba *et al.*, 1971). Acetylcholine accumulation in the CNS can cause ataxia, seizures and coma. These high levels of ACh induce massive neuronal deaths in various brain areas, particularly in limbic and cortical structures. Death from nerve agents is due to paralysis of the diaphragm, airway obstruction from increased tracheobronchial secretions and depression of the CNS respiratory center (Garigan, 1996).

VX is also thought to possibly react directly with receptors of other neurotransmitters, such as norepinephrine, dopamine and GABA. VX appears to have CNS effects that are unrelated to AChE activity and these agents may produce prolonged effects following convulsive doses (Young *et al.*, 1999).

## Toxicity

Symptoms of acute exposure to OPs may include muscarinic, nicotinic and CNS signs. The muscarinic effects include sweating, hypersalivation, bronchoconstriction and increased bronchial secretions, miosis, bradycardia, hypotension, vomiting and diarrhea, and urinary and fecal incontinence. The nicotinic effects include fasciculations, convulsions and weakness of muscles (including the diaphragm). The CNS effects of nerve agents include restlessness, anxiety, headaches, seizures and coma (Garigan, 1996). Effects after inhalation begin within seconds to minutes post exposure. Death can occur within minutes from inhibition of acetylcholinesterase function.

The “G” nerve gases do not readily penetrate intact skin, but toxicity significantly increases if the skin becomes permeable. Dermal toxicity of VX is high, even through intact skin as the liquid does not evaporate quickly (Berkenstadt *et al.*, 1991; Sidell *et al.*, 1997). With dermal exposures, a very small drop on the skin may cause sweating and fasciculations at the site, starting within 18 hours of exposure. A larger drop may cause loss of consciousness, seizures, apnea and flaccid paralysis, with effects beginning within 30 minutes (Sidell *et al.*, 1997). Liquid tabun in the eye can result in death nearly as quickly as an inhaled dose (EPA, 1985c).

On a per weight basis, toxicity in descending order is: VX > soman > sarin > tabun. As used, sarin is the most potent of the “G” nerve agents and VX is about three times more potent a respiratory agent than sarin. VX is

TABLE 8.5 LD<sub>50</sub> (mg/kg) for various nerve agents

Species	Route	Sarin	Soman	Tabun	VX
Mouse	IP				0.050
	Dermal	1.08	7.8	1	
Rat	IM		0.089		0.012
	SQ			0.162	
	Oral	0.55		3.7	
	Dermal			18	
Human	IM		0.062		0.14
	Dermal	28	5	14	

RTECS (2006) and Sidell *et al.* (1997).

300 times more lethal than tabun on skin (Sidell *et al.*, 1997). See Table 8.5 for LD<sub>50</sub>s of the various nerve agents.

Plasma cholinesterase values usually recover in a few days or weeks, due to the irreversible nature of organophosphate inhibition. RBC AChE recovers more slowly (several days to 4 months) depending on the severity of the depression (Grob, 1956). Delayed neurotoxicity has not been reported in humans following nerve agent exposure. However, delayed peripheral neurotoxicity has been reported in animal studies. Soman, at a dose of 1.5mg/kg, produced severe delayed neuropathy in the atropinized hen assay (Willems *et al.*, 1984). No pre-natal mortality or fetal toxicity was noted in soman-poisoned rats or rabbits, even at doses producing significant maternal toxicity, but other nerve gases showed post-implantation mortality and fetotoxicity (HSDB, 2005; RTECS, 2006).

## Treatment

Remove animal from the toxic environment. Administer oxygen if needed. Intubation and ventilation may be necessary if signs progress. Flush eyes with copious amounts of tepid 0.9% saline or water for at least 15 minutes. Reactive skin decontamination lotion (RSDL) appears to be the best available dermal decontamination agent (Hanssen *et al.*, 2006). If RSDL is not accessible, wash all exposed animals three times with soap and water. The use of a dilute bleach solution (1:10 with water), ethanol or tincture of green soap may be more efficacious (Cancio, 1993).

Emesis is not recommended in oral ingestion due to the rapid development of signs. Activated charcoal might provide benefits even after a topical exposure. Control seizures with diazepam, methocarbamol or barbiturates as needed before proceeding with other treatments.

Atropine sulfate is a reversal agent. It is used for the treatment of muscarinic effects of nerve agent poisoning, but will not reverse nicotinic effects (muscular fasciculations and weakness). Atropine does not affect the

AChE–insecticide bond, but blocks the effects of accumulated acetylcholine at the synapse. Atropinization should be continued until the nerve agent is metabolized (Midtling *et al.*, 1985). Effects of overdosing with atropine include hyperthermia, tachycardia, inspiratory stridor, irritability and dilated and unresponsive pupils (Meerstadt, 1982).

Pralidoxime (2-PAM) can be used to treat the nicotinic signs. Pralidoxime is probably most effective when administered in the first 1 to 3 hours. Pralidoxime is not as effective in the treatment of soman poisoning, due to the quick “aging” (within minutes) of the compound (Sidell *et al.*, 1997). Since VX-inhibited cholinesterase ages slowly, administration of 2-PAM chloride is effective in reactivating the enzyme for up to 48 hours after exposure (Sidell and Groff, 1974).

In Belgium, Israel, the Netherlands, Scandinavia, Portugal and West Germany, obidoxime dichloride (Toxogonin, LUH6) is the favored oxime. It may be a less toxic and more efficacious alternative to pralidoxime in poisonings from organophosphates containing a dimethoxy or diethoxy moiety (De Kort *et al.*, 1988). HI-6 is an alternative oxime that has excellent acetylcholinesterase regenerating action with VX and very good action with sarin (GB). It has a good response to soman, but provides poor to no response following tabun exposures (Hoffman, 1999). HI-6 is given in conjunction with atropine and diazepam (Kusic *et al.*, 1991). Recent experimental evidence suggests that oximes of K series are very effective against OP nerve agents, although their potency in clinical trials has yet to be tested (Kuca *et al.*, 2009a, b).

With human exposures to nerve agents, autoinjectors (AtroPen<sup>®</sup>, Mark I<sup>®</sup>, Combopen MC<sup>®</sup>) are available for use. Most available autoinjectors combine atropine and pralidoxime. Autoinjectors are not used in veterinary medicine as they are not adaptable for different sized patients.

The “G” agents have been detected in urine and water using capillary gas chromatography (GC) and GC-MS (Okudera *et al.*, 1997; Kientz, 1998). Immunoassay has been used to detect VX in biological samples (Ci *et al.*, 1995). Nerve agent detection is not as clinically relevant as the measuring of acetylcholinesterase (AChE). AChE activity can be used as a diagnostic indicator or a screening test. The test can be run on plasma, serum or whole blood. AChE activity that is <70% of normal is generally associated with severe symptoms (Midtling *et al.*, 1985). Blood AChE does not always correlate well with clinical signs and poisoning has been diagnosed in patients with “normal” values.

## Concluding remarks

The “G” agents evaporate and disperse over several hours, they are nonpersistent in the environment (Garigan,



1996). In contrast, VX is an oily liquid that can remain in the environment for weeks or longer after being dispersed (Garigan, 1996; Sidell *et al.*, 1997; Munro *et al.*, 1999; Budavari, 2000). Environmental persistence is estimated to be 0.5–1 day for tabun, 1–2 days for soman, 5 days for sarin and several weeks for VX. Environmental cleanup of organophosphate spills depend on changing the pH to promote hydrolysis to inactive phosphate diester compounds (EPA, 1978). Contaminated soil can be treated with either alkaline substances (sodium carbonate, sodium bicarbonate, calcium hydroxide, calcium hydroxide and calcium carbonate) or chlorine-active compounds (sodium hypochlorite or calcium hypochlorite) (EPA, 1975).

Veterinarians and staff can be dermally exposed to contaminated animals. Rubber gowns, aprons and gloves along with respiratory protection must be worn. Once the animals are bathed, the risk of secondary contamination is low. Leather absorbs organophosphates and is extremely difficult to decontaminate. Leather collars, muzzles and other items should be incinerated.

The production of nerve agents is beyond the capabilities of most terrorist groups. Production requires a significant background in chemistry and outlay of capital. The production of sarin by the Aum Shinrikyo cult was estimated to take 1 year to make, involved 80 persons led by a PhD-level scientist and cost about \$30 million (Leitenberg, 1999).

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# Regulatory considerations in veterinary toxicology

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**Disclaimer:** This chapter was written by Lynn O. Post in his private capacity. No official support or endorsement by the FDA is intended or should be inferred.

included in the list of references for the reader to consult more complete and/or updated information from Internet home pages of the respective federal agencies and other reliable organizations.

## INTRODUCTION

This chapter is organized into six sections: Section I: Food and Drug Administration and the Center for Veterinary Medicine; Section II: Environmental Protection Agency; Section III: U.S. Department of Agriculture; Section IV: Drug Enforcement Administration; Section V: Occupational Safety and Health Administration; and Section VI: Statutes, Regulations and Guidelines. Sections I through V describe the federal agencies that are important to animal health, including processes, organizational structure and mission, and selected programs. Testing methods and pharmacovigilance for the Center for Veterinary Medicine, the Environmental Protection Agency and the U.S. Department of Agriculture are described in Sections I through III. Pharmacovigilance is the emerging area of monitoring for unintended harmful effects of marketed animal health products.

This chapter is an overview of a complex and dynamic area that is under the constant influence of competing interests (consumer groups, industrial groups and professional organizations), new legislation and legal precedents, as well as a new generation of animal health products and issues. Appropriate Internet addresses are

## SECTION I. FOOD AND DRUG ADMINISTRATION AND THE CENTER FOR VETERINARY MEDICINE

### Creation and organization of the FDA and CVM

Animal drugs and medical devices are regulated by the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) under the Federal Food, Drug and Cosmetic Act (FFDCA). The FFDCA and FDA are widely recognized as the preeminent regulatory legislation and agency in the nation. The first Federal Food and Drug Act was passed in 1906. It prohibited interstate shipment of adulterated or misbranded food, drink and drugs, and was administered by the U.S. Department of Agriculture's (USDA) Bureau of Chemistry. In 1938 landmark broader legislation was passed requiring manufacturers to provide evidence of product safety before distributing new drugs. This legislation also provided authority to conduct manufacturing site inspections, establish tolerances for unavoidable contaminants and use court injunctions as enforcement tools. In 1940, FDA was transferred from USDA, ultimately becoming part



TABLE 9.1 Recent major animal health product-related amendments to the FFDCA

Amendment	Summary of changes
Generic Animal Drug and Patent Term Restoration Act (GADPTRA)	Enacted 1988.  Provides for approval of generic copies of animal drug products that have been previously approved and shown to be safe and effective. Provided for prescription status for animal drugs.
Animal Drug Use Clarification Act of 1994 (AMDUCA)	Allows veterinarians to prescribe extra-label uses of approved animal drugs and approved human drugs for animals under certain conditions. Allows FDA to restrict extra-label use in certain circumstances. Allows FDA to establish a safe level for a residue for such extra-label use by regulation or order and require the development of analytical methods for residue detection.
Animal Drug Availability Act of 1996 (ADAA)	Amended the definition of substantial evidence of effectiveness. Created a new category of drugs – “Veterinary Feed Directive Drugs.” Permitted a range of doses to appear on animal drug product labeling, rather than one optimum dose. Provided for Licensing of feed mills and eliminated the requirement for medicated feed applications.

of the Department of Health, Education and Welfare (DHEW), which became the Department of Health and Human Services (DHHS). During the early 1950s, a veterinary branch was established within the FDA's Bureau of Drugs. Subsequently, two major amendments to the FFDCA were passed. The Food Additive Amendments of 1958 expanded FDA's authority over animal food additives and drug residues in animal-derived foods. The Kefauver-Harris Drug Amendments of 1962 brought major changes to the FFDCA, requiring manufacturers to test new drugs for effectiveness before marketing them, and to report adverse drug events promptly to FDA. These requirements were also applied through a modified process to products already on the market. Recognizing the importance of animal health products the FDA established the Bureau of Veterinary Medicine (all Bureaus became Centers in 1984) in 1965. However, animal drugs were still regulated under three different sections of the FFDCA, either as new drugs, antibiotics or if labeled for food-animals, as food additives. Because this situation produced a regulatory scheme for animal drugs that was confusing and more complex than for human drugs, the Animal Drugs Amendments of 1968 were passed consolidating the various sections of the FFDCA that applied to animal drugs in one section (512) of the FFDCA. More complete discussions of the history of the FFDCA and CVM are available (Teske, 1995; FDA Center for Veterinary Medicine, 1999). More recent animal health product-related amendments to the FFDCA are given in Table 9.1 (FDA Center for Veterinary Medicine, 2000).

FDA is an agency within the DHHS. The senior official within FDA is the FDA Commissioner. The agency is organized by product responsibility into Centers: Center for Veterinary Medicine (CVM); Center for Drugs Evaluation and Research (CDER); Center for Biologics

Evaluation and Research (CBER); Center for Devices and Radiological Health (CDRH); and the Center for Food Safety and Nutrition (CFSAN). The FDA is also organized geographically into Regional Offices and component Districts where field staff are assigned.

### Approving and monitoring animal drugs

Under the FFDCA, the CVM is responsible for ensuring that animal drugs and medicated feeds are safe and effective for their intended uses and that food from treated animals is safe for human consumption. To accomplish its mission, the CVM is organized into pre-market review, post-market monitoring and research components. In addition, FDA field staff conduct inspections of FDA-regulated animal health industries and products. Prior to a product being marketed it must have an approved New Animal Drug Application (NADA). In order for an NADA to be approved there must be substantial evidence that the new animal drug is effective and safe for its intended uses under the conditions of use prescribed, recommended or suggested in the proposed labeling. In addition, methods, facilities and controls used for the manufacturing, processing and packaging of the drug must be shown to be adequate to preserve its identity, strength, quality and purity (Good Manufacturing Practices, 21 CFR 210 and 211). For food-animal products safety includes safety of drug residues in animal-derived food. Tolerances for residues of new animal drugs in food may be found in 21 CFR 556. Safety would also include an assessment of the effects of the proposed animal drug on the environment and on human health. Following approval, products are monitored to ensure safety and effectiveness and maintained under actual conditions of use.

## Testing methods for animal drugs

FDA has developed extensive detailed guidelines for the conduct of studies required to obtain approval to market products. This section provides a brief description of the animal safety and efficacy studies and human safety studies that may be performed. They are provided to familiarize the student with the general scope of information required for product approval, not to provide guidance in designing studies for product approval. The specific pertinent agency guideline should be consulted prior to designing a study protocol.

### **CVM target animal safety and product efficacy testing requirements**

#### *Introduction*

A veterinary drug sponsor must show that a drug is safe to use as described in the proposed labeling (21 CFR 514.1(b)(1) and 512(d) of the FFDCA; [FDA Center for Veterinary Medicine, 1989](#)). The information needed to determine a products' safety in the target species depends on several factors: proposed use, type of product, chemistry, intended species, breed and class of animal (calves versus mature ruminants), claims and previous use history. Existing data collected from previous studies are used to develop and refine protocols for toxicity studies. While the CVM target animal safety guideline is intended to provide general directions for acquiring essential information, all the data requirements for a standard target animal safety study are not required for every product, and special circumstances may require the collection of additional data that is not specified for the standard study.

#### *Good laboratory practices and general study requirements*

Laboratory studies in animals are conducted according to Good Laboratory Practice (GLP) regulations (21 CFR Section 58). Target animal safety studies are also subject to GLP regulations. The GLP regulations ensure that methods and procedures for collecting, processing and reporting data are standardized and verified for the entire study. GLPs ensure an adequate level of accuracy and quality control. Many GLP requirements are inherent in any well-designed scientific study. On the other hand, adherence to GLP standards does not guarantee the scientific validity of the study.

Since animal husbandry requirements often differ for laboratory animals and domestic animals, domestic animals utilized in clinical studies are not maintained under GLP conditions. Appropriate diagnostic tests, vaccinations, prophylactic and therapeutic treatments are completed prior to the treatment phase of the study. During this initial phase a complete physical examination is performed and baseline data are collected by a

qualified person. The test product formulation must be identical to the proposed marketed product. The route of administration should be the same as the proposed labeling; however, the toxicity or nature of the product may require a different formulation. For example, due to feed refusal, a drench of a product may be required instead of delivery of the drug through a medicated feed.

Data are collected at predetermined intervals during the trial. Clinical observations are recorded at specified intervals, usually twice a day, 7 days a week during the entire study or modified according to the study protocol. During the study appropriate clinical pathology procedures are conducted in all test groups, usually only half of the animals selected at random from each group at predetermined intervals. Histopathologic examinations may be required from all or a portion of the animals. Tissues from the highest treatment and control groups must be examined. If microscopic lesions are observed, corresponding tissues from the next lower treatment group are examined until a no observable effect level (NOEL) is established.

#### *Drug tolerance test*

Under controlled conditions, the target animal response to a toxic dose is characterized by clinical signs. Clinical signs include changes in behavior and appearance and gross lesions in animals that die during the study. Clinical pathology and histology data reveal physiological functions that are most readily affected by the drug. The duration of the toxicity studies may range from acute to chronic.

Target animal safety studies require systemic testing for new products or new chemical entities and additional animal species. They are not required for drugs administered locally, such as otic, ophthalmic, intramammary and intra-articular preparations. This does not apply to generalized dermal or topical drugs that may act systemically. The market formulation of a drug must be administered according to the proposed conditions of use. If volume or palatability becomes a limiting factor for administration, gavage, intravenous, multiple sites or incremental doses may be used. A vehicle control group must be included in the experimental design.

Usually, up to ten times (10×) the maximum proposed clinical dose is administered. The toxic dose (10×) may be reduced or increased in order to manifest toxic signs without causing death. The main purpose of the tolerance test is to characterize toxic signs which can be used to develop toxicity study protocols at lower doses (1×, 3× and 5×). The toxicity studies are used to design the clinical efficacy study protocol.

#### *Toxicity study*

The objectives of toxicity studies in target animal species are: (1) to demonstrate safety of the drug product under

condition of use, (2) to demonstrate signs and effects associated with toxicity and (3) to demonstrate a margin of safety at 5× or below ([FDA Center for Veterinary Medicine, 1989](#)). If the drug has a narrow margin of safety with an intended use in debilitated animals, safety studies in diseased animals may be necessary. This may require more animals per treatment group because of greater variability. Special studies may be requested by FDA, such as lameness, reproductive, dermal irritation, specific routes of administration, tissue disposition and combination drugs.

### ***FDA-required toxicological testing in food-producing animals***

#### *General considerations*

Studies must be conducted according to GLPs. For compounds used in food-producing animals, FDA is concerned with intermittent and chronic exposure of people to relatively low concentrations of residues. FDA tailors the type of toxicological testing needed to show safety for a specific compound. Factors, which are considered include the proposed use, the potential human exposure of the parent compound and/or its residues, as deduced by structure–activity relationships. Some compounds need only a minimum of testing; others may need very extensive studies in a number of diverse biological systems.

The purpose of the toxicology studies is to define the biological effect(s) of the sponsored compound and its quantitative limits. FDA generally asks that the sponsor (owner of the NADA), at a minimum, determines a dose of the compound that produces an adverse biological effect in test animals and a dose that does not produce any significant toxicological or pharmacological effect (NOEL). The spacing of the doses should provide an assessment of the dose–response relationship ([FDA Center for Veterinary Medicine, 2005](#)).

#### *Testing requirements*

FDA recommends the following studies as the minimum necessary for each sponsored compound:

- A battery of genetic toxicity tests.
- A 90-day feeding study in both a rodent species (usually the rat) and a non-rodent mammalian species (usually the dog).
- A two-generation reproduction study with a teratology component in rats. Two litters should be produced in each generation. If toxicity occurs at a lower dose, with a higher incidence, or with greater intensity in the second generation as compared to the first, then the study should include a third generation.

FDA may require the following additional studies, which should be conducted using an approved protocol:

- Chronic bioassays for oncogenicity in each of two rodent species when indicated by positive results in genetic toxicity tests.
- One-year feeding studies in a rodent species (usually the rat) and in a non-rodent species (usually the dog) are needed, when human residue exposure exceeds 25 µg/kg body weight/day. This daily intake is equivalent to 1 part per million in total solid diet of 1500 g, normally consumed by a 60 kg human subject. The FDA will calculate permissible exposure, when evidence indicates that the residue bioaccumulates in the tissues of target animals.
- A teratology study in a second species when the compound is structurally related to a known teratogen, when the compound has hormonal activity that may affect the fetus or when the compound shows adverse effects in the reproduction/teratology study indicating that the compound may be a teratogen.
- Other specialized testing if necessary to define the biological effect of the compound. Examples of specialized studies include testing for neurotoxicity, for immunotoxicity, for hormonal activity, for toxicity following *in utero* exposure or for toxicity from a “bio-mass” product.

If the testing shows that the sponsored compound is a carcinogen, FDA will apply the “no-residue” requirement of Section 409(c)(3)(A), 512(d)(1)(H) or 706(b)(5)(B) of the Act as operationally defined in 21 CFR subpart E of Part 500. FDA will calculate the concentration of residue giving no significant risk of cancer from the tumor data using a statistical extrapolation procedure. In the absence of information establishing the mechanism of carcinogenesis for a particular chemical, FDA will use a non-threshold, linear-at-low-dose extrapolation procedure that determines the upper limit of the risk ([Gaylor and Kodell, 1980](#); [Farmer \*et al.\*, 1982](#)). In the extrapolation FDA will use the upper 95% confidence limit on the tumor data and a permitted maximum lifetime risk to the test animal of 1 in 1 million.

#### *Acceptable daily intake and safety factors*

For other toxicological endpoints, FDA will calculate the acceptable daily intake (ADI) from the results of the most sensitive study in the most sensitive species. From that study the ADI is the highest dose showing NOEL divided by the appropriate safety factor ([Table 9.2](#)).

#### *Sex steroids*

Although not all sex steroids are demonstrated carcinogens, the evidence supports FDA’s conclusion that all

**TABLE 9.2 CVM: human food safety studies and safety factors**

Type of study	Safety factor
Chronic	100
Reproduction/teratology (100 for a clear indication of maternal toxicity, 1000 for other effects)	100 or 1000
90 days	1000

**TABLE 9.3 CVM: human food safety and endogenous sex steroids**

Sex steroids	Daily production ( $\mu\text{g}$ )	Permitted increased exposure ( $\mu\text{g}$ )
Estradiol	6	0.06
Progesterone	150	1.50
Testosterone	32	0.32

endogenous sex steroids and synthetic compounds with similar biological activity should be regarded as suspect carcinogens, and that the endogenous sex steroids are not genotoxic agents. If these compounds produce an oncogenic response in experimental animals, the mechanism of action is not related to a direct chemical interaction with DNA. Specifically, tumor development is a consequence of hyperproliferation of endocrine-sensitive tissue resulting from persistent overstimulation of the hormonal system.

The safety of endogenous sex steroids and their simple ester derivatives can be assured without additional animal study data. Large quantities of these compounds are produced by *de novo* synthesis in people and food-producing animals. The FDA has concluded that no physiological effect will occur in individuals ingesting animal tissues that contain endogenous steroids equal to 1% or less of that produced daily in the population with the lowest production. In the case of estradiol and progesterone, prepubertal boys synthesize the least; in the case of testosterone, prepubertal girls synthesize the least. The daily production values and the calculated increase permitted above the amount naturally present in untreated target animals are listed in Table 9.3. The product is considered safe within the meaning of the Act, if data acceptable to the FDA demonstrate that under the proposed conditions of use, the residue concentration of the endogenous sex steroid in treated food-producing animals does not exceed the permitted increase at the time of slaughter.

#### *Synthetic sex steroids*

In addition to the standard requirements, FDA recommends the following additional tests:

- A 180-day study in rhesus monkeys or another suitable subhuman primate is required to assess the effect

of the sponsored compound on various parameters including ovulation, menstrual cycle, circulating levels of gonadotropins and sex steroids. The study should establish a dose that gives a no observed hormonal response.

- FDA will also normally use a safety factor of 100 for the study in subhuman primates. If a carcinogenic response is observed in a non-endocrine-sensitive tissue, FDA will determine the dose that will satisfy the “no-residue” requirement of the act using the tumor data from that tissue and a statistical extrapolation procedure.

### **Animal efficacy studies**

Efficacy studies are clinical studies. The purpose of an efficacy study is to evaluate the response of the test article under actual conditions of use. Clinical studies to establish efficacy are also important to the overall safety assessment of the product. GLP regulations do not apply because clinical studies are conducted in a clinical environment under actual conditions of use, rather than in a rigorously controlled laboratory setting. For example, veterinary practitioners may enroll their client’s dogs in a non-steroidal anti-inflammatory study and food-animal producers may participate in a production drug study. In efficacy studies, special care must be exercised in handling animals to avoid undue stress which may alter the response to the drug, greatly affecting the study results. If there is a need to collect samples for laboratory analysis that is unrelated to the collection of efficacy data, all animals are sampled at specified times in order to minimize bias.

The design of the efficacy trial is flexible and depends on the species, breed, drug, class of production animal and data endpoints. In some instances, such as for production drugs, many animals are required per treatment group for the efficacy trial because of variability due to environmental conditions and in the test animals. For typical therapeutic products, the number of subjects required for an animal drug clinical trial is about 5–10% of the number required for an equivalent human drug clinical trial. Additionally, while toxicity is not the objective of these studies, animals are observed periodically (usually daily) for clinical signs and abnormalities. These observations give valuable information for designing additional safety study protocols or reveal information that would appear in the safety information on the product label. Some animals may be necropsied at the end of the study if animals exhibit signs of toxicity. On the other hand, the drug may also be discontinued in an animal exhibiting toxic signs.

Even though the efficacy studies are non-GLP, the test methods and procedures for administering the test article are verified and monitored throughout the study. This ensures that the drug is being delivered to the animals according to the protocol. For example, an oral



drug may be administered with or without food, or the mixing procedure for dispersing a production drug in feed must be periodically validated.

After all requirements have been met, FDA approval of the product allows it to be legally marketed and promoted as a new animal drug. The label must contain the product claim(s), pharmacology, side effects, precautions and warnings. After approval, the drug is monitored through periodic and special industry drug experience reports (DERs) to CVM. These reports include adverse experiences and may result in label changes related to product safety.

### ***Regulatory requirements for reporting animal adverse drug events***

#### *Introduction*

CVM does not evaluate complaints for pesticides and animal vaccines. These complaints should be sent to the Environmental Protection Agency (EPA) or the USDA. The CVM will accept safety information for animal feeds, animal devices, human drugs used in animals and approved animal drugs. Mandatory reporting of safety complaints by the company are only required for approved animal drugs to include medicated feeds. Animal medicated feeds may be mixed incorrectly or given to the wrong species, such as when monensin intended for cattle is fed to horses. Alternately, animal feeds may be contaminated with natural or man-made toxins, such as aflatoxin and polychlorinated biphenyls (PCBs), with the potential for serious consequences in humans and the target species.

Although there are no regulatory requirements for the firm to report post-approval safety information for animal devices, CVM will take regulatory action when necessary. Pre-market approval is not required: the FDA does not require formal pre-market approval for devices used in veterinary medicine (510(k) of the FFDCa). Firms that manufacture radiation emitting devices need to register their products under the radiological health regulations, administered by the Center for Devices and Radiological Health. FDA does have regulatory oversight over veterinary devices and can take appropriate regulatory action if a veterinary device is misbranded, mislabeled or adulterated. It is the responsibility of the manufacturer and/or distributor of these articles to assure that these animal devices are safe, effective and properly labeled.

The Center for Drug Evaluation and Research does not require the pharmaceutical company to report safety complaints from extra-label use of human drugs in animals. CVM will process and evaluate the complaints concerning the extra-label use of human drugs in animals, such as methimazole use for hyperthyroid cats.

#### *Pharmacovigilance*

Pharmacovigilance consists of the means and methods for monitoring and ensuring the safety and efficacy of

marketed medicinal products. (*Note:* For the purpose of this section, the term “pharmaco-” is used to encompass not only drugs, but all types of medicinal products.) Pharmacovigilance is made necessary by the limitations of pre-approval or pre-registration studies for animal medicinal products. Economic and practical considerations limit the number of animals included in safety and efficacy studies. Further, many of the animals utilized during the testing phase consist of experimental animals that are homogeneous in age, breed and genetics. More variation is obtained when the drug is tested in clinical studies, but these types of studies generally have few animals. Thus, while pre-marketing studies may be adequate in demonstrating efficacy and common adverse drug reactions, they generally have limited power to detect less frequently occurring adverse events that may occur when the product is finally utilized under actual conditions of use in the general veterinary community.

Veterinary pharmacovigilance within the federal government is typically based on the receipt of spontaneous reports involving complaints of product performance. Spontaneous refers to reports that are voluntarily submitted by veterinarians, animal producers and animal owners. The reports may be submitted by mail, phone, fax or email. The reports may be originally submitted to either the drug company involved or directly to CVM. Product complaints might involve a suspected animal injury, failure of the product to perform (or ineffectiveness) or a product defect.

The key to understanding the spontaneous AER (adverse event report) reporting process is in recognizing and appreciating the word “suspected.” Any complaint must start with the establishment of an actual AER. The pharmacoepidemiologist understands that any one spontaneously reported AER might be misrepresented, or perhaps not existed at all. A certain level of bias is automatically introduced when the reporter associates the AER with the administration of a specific drug. Lacking evidence that unquestionably documents and supports the AER occurrence, information criteria must be established for accepting an AER into a pharmacovigilance system. This information should include an identifiable reporter, an identifiable animal, an identifiable product and an adequately described adverse event.

In the case of the existence of an actual AER, a reporter lodges a complaint in the form of an AER. The AER is the mechanism through which all relevant aspects of the AE are described. The main goal of the pharmacovigilance program receiving an AER should be to ensure that all needed data elements are accurately and fully described. In many cases, follow-up information may be needed to augment the original AER. Even if the most accurate information is obtained for an AER, the possibility of one or more alternative non-drug-related explanations for the AE occurrence cannot be discounted.

This type of system establishes some level of assurance that the drug under question was “associated” with the reported AER. Strength of association is often determined by utilizing objective-based guidelines termed algorithms. The main strength of the spontaneous reporting system is in detecting the occurrence of drug-related adverse effects that are uncommon and cannot be detected by pre-marketing studies, which generally have low statistical power. Once a trend or suspected adverse reaction is detected, other methods may be used to investigate the suspected AER and determine the most appropriate course of action. For instance, the drug company may be requested to provide more information or to conduct studies investigating the specific issue, or expert opinions may be sought from relevant veterinary specialists. Recommended actions might include labeling changes, the recall of specific batches of product or even the removal of the product from the market.

*FDA/CVM adverse drug event reporting system for approved animal drugs*

The regulations that specifically address the spontaneous reporting obligations of the drug sponsors of FDA-approved animal drugs are contained in “Records and reports concerning experience with new animal drugs for which an approved application is in effect” (21 CFR 514.80). Reporting of AERs is mandatory for the pharmaceutical industry. It is voluntary for veterinarians and consumers.

For industry reporting, the regulations categorize AERs into three categories: significant product defect reports that should be submitted to an FDA District Office within 3 days (21 CFR 514.80(b)(1)); AERs involving unexpected animal injury and unexpected product ineffectiveness that should be submitted within 15 working days to CVM (21 CFR 514.80(b)(2)); and the remaining types of AERs and product defects which should be submitted at periodic intervals to CVM (21 CFR 514.80(a)(4)). All categories of complaints are required to be submitted by the drug sponsor on the OMB-approved Form FDA 1932 (“Veterinary Adverse Drug Reaction, Lack of Effectiveness, and Product Defect Report”).

Significant product defects are those involving either label mix-ups or a significant departure of the product from approved specifications, where the product defect may result in immediate harm to animals. Corrective action is accomplished through the FDA Field Office responsible for the manufacturing site.

AERs requiring 15-day submissions are serious and unexpected (21 CFR 514.3 and 514.80):

- fatal or life threatening;
- require professional intervention;
- causes an abortion, or stillbirth, or infertility, or permanent disability disfigurement;

- unexpected AERs are not on the current label or may be pathophysiologically related to an AER on the label.

Unexpected refers to information that is not contained either on product labeling or as part of the approved FDA application. Regulations further require reporting of unexpected adverse events that are associated with clinical use, studies, investigations or tests, whether or not determined to be attributable to the suspected drug. A key phrase is whether or not it is determined to be attributable to the suspected drug. CVM expects the drug sponsor to submit all AERs rather than only those reports the firm believes are associated with the AER. CVM considers selective report submission (filtering) to introduce a bias that confounds the evaluation of submitted AERs.

All AERS submitted to CVM are evaluated by a staff of veterinarians assigned to this task. All relevant information is extracted from the AERs and entered into the appropriate fields of a relational database, using standard terminology. A six-step algorithm evaluation process is utilized to assign a score that represents the strength of the veterinarian’s opinion that the use of the drug was associated with the adverse clinical sign.

The database is utilized by product manager veterinarians assigned to monitor the safety and efficacy of specific products. In the event that a potential problem is identified, the Monitored Adverse Reaction Committee (MARC) is convened to assist in evaluating information related to the potential problem. The MARC also recommends appropriate regulatory action, in the event the problem is considered significant. AER information is released to the public by means of summary report publication on the CVM website. Additional requests for AER information can be made through the freedom-of-information officer.

AER submission is required only from companies marketing FDA-approved animal drugs. There are no requirements for submitting AERs for human drugs used in animals or unapproved products labeled for animals. CVM will accept reports directly from consumers and veterinarians regarding extra-label use of human drugs and unapproved animal drug products. CVM has limited ability to monitor the performance of these drugs in animals.

Veterinarians are encouraged to report AERs directly to the manufacturer/sponsor. The manufacturer should record the information and send a report of their investigation to CVM. Another reporting option is to call the CVM AER hotline (1-888-FDA-VETS) to report AERs. An FDA veterinarian will return the phone call. The reporter can also either call or write the CVM to obtain a pre-postage-paid reporting form.

AER reporting to CVM has significantly increased over the last decade. During the early 1990s, the CVM

received approximately 1000 AERs each year. CVM received about 33,000 AER reports for the year 2005. Reasons for the increase are numerous, but include the new types of drugs approved for use in companion animals, label information provided for contacting drug companies and the interest of the public in reporting perceived product problems.

#### *The future of global pharmacovigilance reporting*

The USDA and FDA are participants in the construction of a global AER platform. The reporting format for AERs will be standardized but not the causality assessment, evaluation of risk for each AER and the pharmacoepidemiologic approach that may include a mathematical analysis. Standardized evaluation of AERs is impractical because conditions of use for a drug vary from country to country. Spontaneous reporting does not readily lend itself to classical epidemiologic analyses because the numerator (number of animals) is under-reported and the denominator (number of doses) is unknown. Therefore, it is extremely difficult to calculate a true incidence.

Global harmonization of reporting requirements for AERs is no small task. It must be kept in mind that, unlike human AERs, veterinary medicine deals with multiple species which adds more complexity to a complicated process. In addition, reporting requirements differ substantially from country to country because missions, goals, resources, administrative procedures and AER sources vary considerably. The variations reflect the fundamental differences in the worldwide agencies and the laws they administer. The International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH, 2005) is a trilateral (EU–Japan–United States with two observer countries: Australia and Canada) program aimed at harmonizing technical requirements for animal medicinal product registration to include pre-approval review and post-approval monitoring (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products). In 1996, at the inception of VICH, one goal was the harmonization of pharmacovigilance reporting requirements: definitions, data elements, electronic submission protocols and medical dictionaries.

Successful efforts of VICH will lead to the timely transmission of AER information between all parties and organizations involved in animal pharmacovigilance. Electronic reporting standards would also be available to consumers and veterinarians who could report directly to the regulatory agency rather than the company. The standardized AER information will be disseminated

worldwide for examination and analysis by all interested parties.

## SECTION II. ENVIRONMENTAL PROTECTION AGENCY

### Creation of the EPA

During the 1960s activists became increasingly effective in focusing attention on environmental and public health issues. Rachael Carson's book, *Silent Spring* (1962), provided a worldwide view of the effects of indiscriminate use of pesticides. By 1969, the word "ecology" had become part of American culture. Under criticism that his environmental committees were largely ceremonial bodies, President Nixon appointed a White House Committee in December of 1969 to consider whether there should be a separate environmental agency. About the same time, Congress sent the National Environmental Policy Act (NEPA) to the president, which he signed on January 1, 1970.

It was against the background of environmental activism that Reorganization Plan No. 3 was sent to Congress by the Nixon Administration. Under Reorganization Plan No. 3, the EPA was formed in 1970 to establish and enforce environmental protection standards; to conduct environmental research; to provide assistance to other agencies in combating environmental pollution; and to assist in developing and recommending new policies for environmental protection. The regulatory activities for environmental protection were consolidated from 15 components of five executive departments and independent agencies into a single agency, the EPA. Certain pesticide regulatory functions were transferred from FDA and USDA to EPA. The enactment of major new environmental laws and important amendments since 1970 have expanded and refined the role of the EPA (Table 9.4).

The EPA is organized into major Offices headed by assistant EPA administrators which develop and implement EPA's policies and programs. For instance, the Office of Prevention, Pesticides and Toxic Substances (OPPTS) develops national strategies for toxic substance control and promotes pollution prevention and the public's right to know about chemical risks. A component Office, the Office of Pesticide Programs (OPP), regulates the use of all pesticides in the United States and establishes maximum levels for pesticide residues in food. The EPA also has geographically organized regions. Each EPA Regional Office is responsible, within selected states, for the execution of the Agency's programs, considering regional needs

**TABLE 9.4 Major statutes that form the basis for the programs of the EPA**

Statute or law	Description
Federal Food, Drug, and Cosmetic Act, Delaney Clause (1958)	FFDCA prohibited any man-made chemical in food that caused cancer in any animal, including man.
National Environmental Policy Act (1969)	NEPA is the basic national charter for protection of the environment.
Clean Air Act (1970)	CAA is the comprehensive federal law that sets standards regulates air emissions from area, stationary and mobile sources.
Occupational Safety and Health Act (1970)	OSHA ensures worker and workplace safety from hazards, such as exposure to toxic chemicals, excessive noise levels, mechanical dangers, heat or cold stress, or unsanitary conditions.
Federal Insecticide, Fungicide, and Rodenticide Act (1972)	FIFRA provided federal control of pesticide distribution, sale and use and required users (farmers, utility companies, and others) to register when purchasing pesticides.
Endangered Species Act (1973)	ESA provides a program for the conservation of threatened and endangered plants and animals and the habitats in which they are found.
Safe Drinking Water Act (1974)	SDWA was established to protect the quality of drinking water in the United States.
Resource Conservation and Recovery Act (1976)	RCRA gave EPA the authority to control hazardous waste during the generation, transportation, treatment, storage, and disposal of hazardous waste.
Toxic Substances Control Act (1976)	TSCA gave EPA the ability to track about 75,000 industrial chemicals produced or imported into the United States.
Clean Water Act (1977)	CWA gave EPA the authority to set effluent standards on an industry basis (technology based) and continued the requirements to set water quality standards for all contaminants in surface waters.
Comprehensive Environmental Response Compensation and Liability Act (1980)	CERCLA created a tax on the chemical and petroleum industries and provided broad federal authority to cleanup abandoned hazardous waste sites (Superfund).
Emergency Planning and Community Right-to-Know	EPCRA was enacted by Congress as the national legislation on Act (1986) community safety.
Superfund Amendments and Reauthorization Act (1986)	SARA amended CERCLA and reflected EPA's experience in administering the complex Superfund program.
Pollution Prevention Act (1990)	PPA encouraged industry to control toxic emissions by using cost-effective changes in production.
Oil Pollution Act (1990)	OPA streamlined and strengthened EPA's ability to prevent and respond to catastrophic oil spills through a tax on oil.
Food Quality Protection Act (1996)	FQPA amended FIFRA and FFDCA.

and the implementation of federal environmental laws (USEPA, 2000b).

### **Pesticides and the federal insecticide, fungicide and rodenticide act**

The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) has a major impact on the practice of veterinary medicine because it provides EPA the authority to regulate pesticide use in animals. Under FIFRA the term pesticide includes products such as insecticides, fungicides, rodenticides, insect repellants, weed killers, anti-microbials and swimming pool chemicals. Pesticides are intended to prevent, destroy, repel or reduce pests of any sort. If a pesticide can be used with "reasonable certainty of no harm," it is granted a "registration" that permits its sale and use according to requirements set by EPA to protect human health and the environment. During

its evaluation of a proposed pesticide the EPA considers the toxicity of the pesticide, the quantity and frequency of pesticide application and the amount of pesticide that remains on the food by the time it is marketed.

The FIFRA was enacted in 1947 as a consumer protection statute to regulate the manufacture, sale, distribution and use of pesticides. The USDA administered FIFRA. Pesticide registration was required before marketing in interstate commerce, and the pesticide product had to bear a label with the manufacturer's name and address, name brand and trademark of the product, net contents, ingredient list, warning statements to prevent injury, and directions for use. Subsequent amendments added more product classes including nematicides, plant regulators, defoliants and desiccants (1959); and a requirement that all pesticide labels contain a Federal Registration Number and safety information words such as "warning," "danger," "caution" and "keep out of reach of children" (1964).



In 1970, authority for FIFRA was transferred from the USDA to the newly created EPA. Another amendment in 1972 provided the EPA with authority to regulate pesticides to prevent unreasonable adverse effects on the environment. The 1972 amendment changes included requirements to follow the label, heavy fines for violations, classification of pesticides as “restricted use” or “general use,” state certification, manufacturer registration and inspection by EPA, and scientific evidence that the product is effective and safe for humans, crops and animals. Subsequent clarifying amendments provided for significant changes to improve the registration process including generic standards for the active ingredient rather than for each formulated product, re-registration of all products registered prior to 1975, conditional registration where efficacy data may be waived, state enforcement authority and defined the phrase: “to use any registered pesticide in a manner inconsistent with its labeling.” In 1988, the EPA was required to re-register existing pesticides that were originally registered before current scientific and regulatory standards were formally established. The Food Quality Protection Act (FQPA) of 1996 is the most recent significant amendment to FIFRA.

## Pesticide residues in foods

When pesticides are used in producing food, they may remain in small amounts called residues. To protect the public from potentially harmful effects caused by pesticide residues on food, the EPA regulates the amount of each pesticide that may remain on or in each food. EPA regulates pesticides under two major federal statutes. Under the FIFRA, EPA registers pesticides for use in the United States and prescribes labeling and other regulatory requirements to prevent unreasonable adverse effects on health or the environment. Under the FFDCA, EPA establishes tolerances (maximum legally permissible levels) for pesticide residues in food. Tolerances are established as part of the pre-market registration process. Exceeding the tolerance initiates enforcement actions where the commodity may be subject to seizure. Tolerance limits apply to both domestic and imported food. Some foods do not require tolerances because the exposure and toxicity data show that the food is safe when the pesticide is used according to the directions on the label.

Several other government agencies enforce EPA’s pesticide tolerances in food. The FDA tests food produced in the United States and imported food. State agencies also test food produced in the United States. The USDA tests milk, meat and eggs. The USDA and FDA have programs designed to develop statistically valid information on pesticide residues in foods. This information is provided to EPA for use in risk assessments for pesticides.

When the USDA detects violations of tolerances in their data collection program, they notify FDA.

EPA has responded to both large and small environmental disasters during the past 30 years. Less visible but just as important is EPA’s present effort to reassess all the pesticide and other ingredient tolerances and exemptions that were in effect as of 1996 when the FQPA of 1996 was passed. It amended both the FIFRA and the FFDCA. These amendments changed the way EPA regulates pesticide residues. Examples of FQPA changes include a single residue safety standard of “a reasonable certainty of no harm”; requirement for an explicit determination that pesticide tolerances are safe for children (includes an additional safety factor); limits consideration of benefits when setting tolerances to non-threshold (such as carcinogenic) effects; requirement to review existing pesticide tolerances within 10 years; authorization to require new data, including potential endocrine effects; imposition of civil penalties; and expedited review of safer pesticides.

## EPA companion animal safety testing

Companion animal safety studies for EPA-registered pesticides apply only to dogs and cats because of widespread use of these external products for pests (USEPA, 1998). The companion animal safety study is required for pesticide registration. Companion animal safety studies for pesticide formulations for the treatment of external pests are intended to demonstrate that an adequate margin of safety exists with overuse or misuse (40 CFR Section 792 and 40 CFR 160, GLP Standards). Data from companion animal safety studies for pesticides serve as a basis for product labeling. The study can be compared to an acute dermal toxicity study, and is limited because an NOEL is not required. External pesticide products include collars, sprays, dips, shampoos and spot treatments.

The design of a companion animal safety study should reflect the product label: method of administration, species and age group, and frequency of application. The criteria for use of the product should be used in the study. A control group should receive a concurrent vehicle at the 5× level. The vehicle should contain the inert ingredients at the maximum levels that would appear in the 5× formulation. Negative (untreated) controls may occasionally be employed to determine whether adverse effects are due to the inert ingredients in a formulation. The test formulation is applied to several groups of (six/sex) experimental animals at the label dose and multiples of this dose (3× and 5×). For exaggerated doses (3× and 5×), products specifically prepared for this type of study that contain higher concentrations of the active ingredient are preferred. If the drug cannot be formulated at the 3× or 5× concentration, due to volume constraints, multiple treatments at frequent intervals may be

necessary. If the high-dose level (at least 5×) produces no evidence of drug-related toxicity, a full study (three dose levels) may not be necessary. Multiple pesticides formulated into a single product may also be evaluated. Depending on the severity of clinical signs of toxicity, products with less than 5× margin of safety may be considered for registration. The route of administration of these product studies should be topical. The skin or hair should not be prepared in any manner unless such directions appear on the label. If the product label recommends several treatments, multiple treatments at frequent intervals are included in the study design based on label claims and instructions for use.

The species recommended for treatment on the product label is included in the study. Studies are performed on representative classes of healthy dogs and cats by size, weight, sex and age based on label claims. For instance, if only adults over 6 months of age are the label population of animals to receive treatment, only adults are enrolled in the study. If the product is registered for puppies and kittens, the label should state a minimum age for this group and this class should be included in the study. An equal number of animals per sex are used at each dosage level. Animals are appropriately examined and prepared during the acclimation period.

Clinical observations are conducted at hourly intervals for at least 4h after the last treatment, and twice daily for the duration of the study. If adverse reactions are observed, the observation period on the day of treatment is extended to a time at which no toxic signs are observed. Observations should include all systems. Special attention is directed to observations of central nervous system signs of seizures, tremors and salivation; and gastrointestinal signs of vomiting and diarrhea. Observations and measurements are reported for a minimum of 14 days post-treatment and longer if appropriate. Various samples and measurements are collected throughout the study. Individual body weights and food consumption are measured during the acclimation and periodically during the study. Animals that die or are euthanized in a moribund state are subjected to a gross necropsy and abnormal tissues are examined histopathologically to determine the cause of death. Routine post-study necropsy is not required. Clinical pathology samples including red cell cholinesterases, when appropriate, are assessed prior to treatment, 24h post-treatment, and on day 7 of treatment.

## Chronic toxicity testing: pesticides in animal-derived food

### Introduction

The USEPA requires a variety of toxicity testing prior to registration of pesticides. The 870 Series Final Guidelines

describe study protocols that meet testing requirements of the FIFRA and the Toxic Substances Control Act (TSCA): Reproduction and Fertility (870.3800) and Combined Chronic Toxicity/Carcinogenicity (870.4300) (USEPA, 1998, 2000a).

### Reproduction toxicity testing

The two-generation reproduction study provides information on the effects of a test substance on the male and female reproductive systems. The study should be conducted in accordance with the GLP Standards stipulated in 40 CFR Part 160 (FIFRA) and 40 CFR Part 792 (TSCA). The test substance is administered to parental (P) animals prior to and during their mating, during the resultant pregnancies and through the weaning of their F1 offspring. The substance is then administered to selected offspring during their growth into adulthood, mating and production, and weaning of a subsequent generation. The rat is the most commonly used species for testing. Each control group should contain a sufficient number of mating pairs to yield approximately 20 pregnant females. Each test group should contain a similar number of mating pairs. At least three dose levels and a concurrent control should be used. The dose levels should be spaced to produce a gradation of toxic effects. The highest dose should be chosen to induce some reproductive and/or systemic toxicity but not death or severe suffering. The intermediate-dose levels should produce minimal observable toxic effects. The lowest-dose level should not produce any evidence of either systemic or reproductive toxicity. The highest dose tested should not exceed 1000mg/kg/day (or 20,000ppm in the diet), unless potential human exposure data indicate the need for higher doses. A concurrent control group should be used. The test substance is usually administered by the oral route (diet, drinking water or gavage). The animals should be dosed with the test substance on a daily basis. Daily dosing of the parental males and females should begin when they are 5–9 weeks old, and that of the offspring should begin at weaning. Daily dosing should begin at least 10 weeks before the mating period and continue until termination.

The endpoints measured should provide data regarding the performance of the male and female reproductive systems including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation and weaning, and on the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, target organs in the offspring and preliminary data on prenatal and postnatal developmental toxicity, and serve as a guide for subsequent tests. Additionally, since the study design includes *in utero* as well as postnatal exposure, it provides the

opportunity to examine the susceptibility of the mature/neonatal animal. For further information on functional deficiencies and developmental effects, additional study segments can be incorporated into the protocol, utilizing the guidelines for developmental toxicity or developmental neurotoxicity (USEPA, 1996).

#### **Combined chronic toxicity/carcinogenicity testing**

The objective of the combined chronic toxicity/carcinogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. If chronic/carcinogenic effects are detected in the first mammalian species, usually the rat, further long-term studies may be conducted in another species, usually the dog. The design and conduct should allow for the detection of neoplastic effects and a determination of the carcinogenic potential as well as general toxicity, including neurological, physiological, biochemical and hematological effects and exposure-related morphological (pathology) effects.

Preliminary studies providing data on acute, subchronic and metabolic responses should be conducted. The rate of exposure is generally oral, dermal or inhalation. For the combined chronic toxicity/carcinogenicity study, the rat is the species of choice for oral and inhalation studies, whereas the mouse is species of choice for the dermal route. The choice of the route of administration depends on the physical and chemical characteristics of the test substance and the product exposure in humans. The duration of studies should be at least 18 months for mice and hamsters and 24 months for rats. The following general requirements apply to all combined chronic toxicity/carcinogenicity studies regardless of the route of administration. Testing should be started with young healthy animals as soon as possible after weaning and acclimatization, but no later than 8 weeks of age. At commencement of the study, the weight variation of animals used should be within 20% of the mean weight for each sex. At least 100 rodents (50 males and 50 females) should be allotted randomly to each dose level and concurrent control group. At least 20 additional rodents (10 males and 10 females) should be used for satellite dose groups and the satellite control group. The purpose of the satellite group is to allow for the evaluation of chronic toxicity after 12 months of exposure to the test substance. The number of animals in any group should not fall below 50% during the course of the study at 15 months in mice and 18 months in rats, so that a meaningful and valid interpretation of negative results can be achieved. Survival in any group should not fall below 25% at 18 months in mice and 24 months in rats.

#### **Organization for Economic Cooperation and Development**

The Organization for Economic Cooperation and Development (OECD) carried forward recommendations

of an international expert group and questioned the value of clinical pathology findings, including hematology and clinical chemistry, beyond 12 months in chronic and combined chronic/carcinogenicity studies (Weingand *et al.*, 1996). As a result, hematology and clinical chemistry measures beyond the 12-month period have been eliminated. The observational and functional tests on the nervous system and behavior have also been revised per OECD. Recently, the requirement for the 6-month long-term studies in the dog has been modified. Long-term studies (6 months or longer) in the dog will not be conducted unless warranted by findings in long-term rat studies.

#### **EPA adverse effects information reporting**

At EPA, regulatory responsibilities for pesticides are centralized at the OPP. Regulatory authority is derived from Section 6(a)(2) of the FIFRA. The regulations that specifically address reporting of adverse effects of pesticides are contained in Reporting Requirements for Risk/Benefit Information (40 CFR Section 159). Reporting of AERs is mandatory for industry.

Pesticides are broadly defined to include insecticides, herbicides, fungicides and various other substances that are not animal drugs used to control pests. A pesticide is also any substance or mixture of substances intended for use as a plant regulator, defoliant or desiccant. The EPA collects information on pesticide use, whether intended or unintended. Adverse effects information may include information on human or animal injury, plant injury or environmental contamination. All adverse effects information is accepted regardless of the registered label use of the product.

Specific industry reporting requirements for adverse effects information depends on the category of the information. Reports of human death should be submitted within 15 calendar days as an individual report. A variety of other information, including suspected product defects, human epidemiology and exposure studies, and pest resistance reports should be submitted within 30 calendar days as individual reports. Reports involving major and moderate human injury, major injury to plants and wildlife, water contamination and public health product ineffectiveness should be submitted after a 1-month accumulation as individual reports.

Animal reports receive less priority than human reports. Reports involving any type of domestic animal injury, and some minor types of human, plant and wildlife injury, should be reported within 2 months, after a 3-month accumulation of reports. The information may be submitted in an aggregate format. An aggregate report for a specific time period includes a count of domestic injuries within categories of assessed seriousness. Information concerning the specific details of each

individual AER is not included in an aggregate. The EPA, however, may request additional information for any AERs that involved the death of an animal.

The EPA/OPP gathers adverse effects information from other sources. Information may result from complaints made directly to the EPA. This AER information is entered into the EPA Incident Database. Information may also be obtained through the National Pesticide Communications Network, based at Oregon State University, and other sources. Much of this information may be entered into the EPA Incident Data System. Some aggregate reports may also be entered into the system.

Various product divisions within OPP review the AER information. A complete review of product-related information is performed when it is being considered for re-registration. A Special Review may be set in motion when EPA has reason to believe that the use of a pesticide may result in unreasonable adverse effects, including acute toxicity to domestic animals. Subsequent regulatory actions may range from removal of the product from the market to changes in permitted uses.

Since the EPA/OPP regulates all pesticides, jurisdictional AER issues resulting from extra-label use are not a consideration. Further, the mission of OPP is to monitor the safety of pesticides in humans and animals regardless of the intended use. Evaluation of domestic animal AERs involving pesticides labeled for therapeutic use on animals is given the same attention as a pesticide labeled for use on plants. Intended use is more likely to affect what regulatory actions may be subsequently taken to improve the overall safety of product use.

## SECTION III. U.S. DEPARTMENT OF AGRICULTURE

### Regulatory authority and mission

The authority for regulating veterinary biologics in the United States is provided in the Virus–Serum–Toxin Act (VSTA), enacted in 1913 and amended in 1985 ([USDA APHIS Center for Veterinary Biologics, 2005a](#)). It requires, with some exceptions, that all veterinary biologics be licensed. The Licensing and Policy Development (LPD) unit in the Center for Veterinary Biologics (CVB), Veterinary Services, Animal and Plant Health Inspection Service (APHIS), and U.S. Department of Agriculture (USDA) enforces the VSTA. The Act authorizes the Secretary of USDA to prescribe regulations governing the preparation and marketing of veterinary biologics shipped into, within or from the United States. The VSTA makes it unlawful to sell worthless, contaminated, dangerous or harmful veterinary biologics or to ship veterinary biologics to or from the United States unless they are prepared in a licensed

establishment in compliance with USDA regulations. It requires the issuance of a permit by USDA prior to the importation of a veterinary biological product and gives the department the authority to test veterinary biological products prior to importation. In case of violation, the Act permits USDA to remove or suspend establishment and/or product licenses and permits. It also gives authority for detention, seizure and condemnation of products and injunctions against products or establishments.

### Definition and functions

The regulations in 9 CFR 101.2(w) define veterinary biological products to be all viruses, serums, toxins and analogous products of natural or synthetic origin that are intended for use in the diagnosis, treatment or prevention of diseases of animals. The products include diagnostics, antitoxins, vaccines, live or killed microorganisms and their antigenic or immunizing components. The LPD reviews license applications for production facilities and biological products and for importation. It reviews production methods, labels and supporting data involved in the licensing and permit process and issues licenses and permits. An exemption from this licensing provision is given for products prepared by: (1) a person solely for administration to that person's own animals; (2) a veterinarian for use in his or her own licensed practice under a veterinarian–client–patient relationship; and (3) a person operating a state licensed facility solely for distribution of product within the state of production in a state that has the state regulatory USDA-approved control program for veterinary biologics. The VSTA provides for the issuance of conditional or special licenses for products in order to meet an emergency condition.

A proposed rule to amend the VSTA (9 CFR Parts 101 and 116) was published in the [Federal Register](#) on August 17, 2005 (Vol. 70, No. 158, p. 48325) ([USDA APHIS Guidance Documents, 2005b](#)). For newly licensed products, the proposed rule requires veterinary biologics licensees to record specific information concerning adverse events associated with the use of biological products that they produce or distribute and to compile and submit those records in a summary report to the APHIS every 12 months for products licensed for one year or more. A summary report would have to be submitted at 6-month intervals during the first year of the product license and at 12-month intervals thereafter. The VSTA did not explicitly require licensees to maintain records of adverse events associated with the use of veterinary biologics, nor do the regulations in Part 116.5 provide specific guidance in determining whether an adverse event should be considered an indication that raises questions regarding the purity, safety, potency, efficacy, preparation, testing or distribution of biologics.



Adverse event and AER are defined as follows:

- Any observation in animals, whether or not the cause of the event is known, that is unfavorable and unintended and that occurs after any use (on or off label) of a biological product.
- For products administered to animals, this would include events related to a suspected lack of expected efficacy.
- AER: as a communication concerning the occurrence of an adverse event from an identifiable first-hand reporter that includes at least the following information: an identifiable reporter; an identifiable animal; an identifiable biological product; and one or more adverse events.

#### *Animal immunobiologic vigilance program*

Regulation of animal immunobiologics is centralized within the APHIS and CVB. Regulatory authority is derived from the VSTA. Animal immunobiologics modulate the immune system for the prevention, treatment or diagnosis of disease in animals. Animal immunobiologics typically include vaccines, toxoids and serum derivatives, which may be used in diagnostic test kits.

Immunobiologic vigilance (or vaccinovigilance) may in some respects be more complicated than monitoring the safety of animal drugs, or even therapeutic animal pesticides. Vaccines are generally utilized for the prevention of disease. The real or perceived failure of a vaccine to perform may be dependent on a number of factors and difficult to assess. Suspected vaccine failure in individuals is particularly difficult to assess. Further, assessing AERs involving adverse events that are manifested some time after vaccine administration can be complicated. In addition to the “active ingredient”, vaccines can include other ingredients, such as preservatives, stabilizers and adjuvants, which can affect the product performance. Vaccinovigilance is a particularly challenging discipline that may require substantially more information from formal studies in order to verify suspected problems.

Until the proposed rule is finalized to amend the VSTA, USDA currently has no regulations that require the immunobiologic industry to routinely submit AERs to CVB. Since the immunobiologic industry does not submit AERs, the CVB has limited ability to monitor AERs involving animal safety. USDA may at times request companies to voluntarily submit AERs that are related to a particular safety issue. Although, the USDA does not presently mandate specific AER record keeping practices, the USDA does have the ability to conduct investigations at the immunobiologic manufacturing site.

At present, modest levels of CVB resources are allocated to direct vaccinovigilance activities. However, CVB

can direct resources, as needed, to safety issues. While regulatory actions may be more likely to result in the removal of the registered product from the market, other actions may include manufacturing changes or label revisions.

#### **Animal care and welfare requirements**

Researchers who conduct studies funded by the federal government are subject to a number of requirements imposed by various federal agencies. The Animal Welfare Act (AWA) is administered by the USDA APHIS Animal Care Program ([USDA APHIS Animal Care Program Publications, 2000](#)). It applies specifically to dogs, cats and a number of other listed species as well as to any other warm-blooded animals designated by the Secretary. It requires covered facilities to register and comply with AWA regulations. Examples of types of requirements established to implement AWA include living space, lighting, heating, ventilation, drainage, transportation, feeding and watering, and veterinary care. It establishes the requirement for an Institutional Animal Care and Use Committee, which must include a veterinarian and an external member. This committee reviews the program, inspects the facility periodically and reviews protocols for research conducted within the facility. It does not apply to animal agriculture or non-research horses. It does apply to dealers and exhibitors. Federal agencies such as National Institutes of Health (NIH) may impose additional requirements.

As a practical approach to ensuring that their programs and facilities meet these requirements, many organizations participate in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's accreditation process ([Association for Assessment and Accreditation of Laboratory Animal Care, 2005](#)). AAALAC was founded in 1965 to address laboratory animal welfare issues. AAALAC International is a non-regulatory, non-profit corporation. Its mission is to promote high standards for animal care, use and welfare, and enhance life sciences research and education through its accreditation process.

## **SECTION IV. DRUG ENFORCEMENT ADMINISTRATION**

### **Mission and public health impact**

Illegal drug use is a widespread problem in the United States. In 1998, an estimated 13.6 million Americans used an illicit drug at least once during the 30 days prior to

being interviewed. During the same period, nearly 1 in 10 youths aged 12–17 years was an illicit drug user, and 130,000 were current users of heroin. The Drug Enforcement Administration (DEA) was established on July 1, 1973. Its mission is to enforce the controlled substances laws and regulations of the United States and to bring to the criminal justice system, or any other competent jurisdiction, those organizations and principal members of organizations who are involved in the growing, manufacture, or distribution of controlled substances in the United States.

### Illicit veterinary drugs of public health concern

Approved veterinary scheduled drugs, which are illicitly used in humans, include boldenone (Equipoise), ketamine, stanozolol (Winstrol) and trenbolone (Finajet). Veterinary products containing anabolic steroids that are exclusively intended for administration through implants to cattle or other non-human species and which have been approved by the CVM, US FDA are excluded from all schedules (Drug Enforcement Agency: 21 CFR Parts 1300–1316, [List of Scheduling Actions and Controlled Substances, 1999b](#); [Drugs of Abuse, 1997](#)).

Ketamine hydrochloride, known as “special k” and “k,” is a general anesthetic for human and veterinary use. Ketamine produces effects similar to pentachlorophenol (PCP) with the visual effects of lysergic acid diethylamide (LSD). Ketamine sold on the streets comes from diverted legitimate supplies, primarily veterinary clinics. Its appearance is similar to that of pharmaceutical grade cocaine, and it is snorted, placed in alcoholic beverages or smoked in combination with marijuana. The incidence of ketamine abuse is increasing. Ketamine was placed in Schedule III of the Controlled Substances Act (CSA) in August 1999 (Drug Enforcement Agency: 21 CFR Parts 1300–1316, [Schedules of Controlled Substances, 1999a](#)).

Concerns over a growing illicit market and prevalence of abuse combined with the possibility of harmful long-term effects of steroid use led Congress in 1991 to place anabolic steroids into Schedule III of the CSA. The CSA defines anabolic steroids as any drug or hormonal substance chemically and pharmacologically related to testosterone (other than estrogens, progestins and corticosteroids) that promotes muscle growth. Most illicit anabolic steroids are sold at gyms, competitions and through mail operations. For the most part, these substances are smuggled into this country. Those commonly encountered on the illicit market include boldenone (Equipoise), ethlestrenol (Maxibolin), fluoxymesterone (Halotestin), methandriol, methandrostenolone (Dianabol), methyltestosterone, nandrolone (Durabolin, DecaDurabolin), oxandrolone (Anavar), oxymetholone (Anadrol), stanozolol (Winstrol), testosterone

and trenbolone (Finajet). Physical side effects include elevated blood pressure and cholesterol levels, severe acne, premature balding, reduced sexual function and testicular atrophy. In males, abnormal breast development (gynecomastia) can occur. In females, anabolic steroids have a masculinizing effect, resulting in more body hair, a deeper voice, smaller breasts and fewer menstrual cycles. Several of these effects are irreversible. In adolescents, abuse of these agents may prematurely stop the lengthening of bones, resulting in stunted growth (Drug Enforcement Agency: 21 CFR Parts 1300–1316, [List of Scheduling Actions and Controlled Substances, 1999b](#)).

### DEA regulatory requirements for veterinarians

Federal DEA regulations are contained in 21CFR 1300 and 1316 ([U.S. Department of Justice, 2005](#)). Clinicians who are authorized to prescribe controlled substances may do so for legitimate medical purposes in the context of a valid veterinary–client–patient relationship. Under these regulations the prescribing practitioner is held responsible in case the prescription does not conform to the regulations for records and reports (21 CFR 1304), submitting proper ordering forms (21 CFR 1305) and for meeting the requirements for prescriptions (21 CFR 1306 (1)). Requirements for prescribing Schedule II drugs are much more stringent than those for Schedules III, IV and V. For example, an oral order or refilling of the prescription for Schedule II drugs is prohibited under the CSA. DEA has divisional offices in various regions, including Atlanta, Boston, Chicago, Dallas, Denver, Detroit, El Paso, Houston and Los Angeles. In addition, each division has several district offices.

## SECTION V. OCCUPATIONAL SAFETY AND HEALTH ADMINISTRATION

### Organization and mission

Harmful chemical compounds in the form of vapors, fumes, mists, gases, liquids and solids are encountered in the workplace by inhalation, absorption (through direct skin contact), or to a lesser extent by ingestion. Airborne chemical hazards have great occupational health significance. The degree of worker risk from exposure to any given substance depends on the nature and potency of the chemicals’ toxic effects and the duration and intensity of exposure ([Occupational Safety and Health Administration, Informational Booklet on Industrial Hygiene, 2005c](#)).

In 1970 Congress passed the Occupational Safety and Health Act to assure so far as possible every worker

in the nation safe and healthful working conditions ([Occupational Safety and Health Administration, the Occupational Safety and Health Act of 1970, 2005a](#)). The Act requires employers to maintain a safe workplace and created the Occupational Safety and Health Administration (OSHA). This agency's mandate includes regulatory responsibility for establishing mandatory occupational exposure standards, as well as related research, training and education and enforcement. OSHA is part of the Department of Labor and is headed by the Assistant Secretary of Labor for Occupational Safety and Health. Major OSHA programs are administered through Directorates such as the Directorate for Safety Standards Programs which provides workplace standards and regulations to ensure safe working conditions.

### *Offices and district offices*

Under the Act many states have OSHA-approved occupational safety and health programs which function *in lieu* of OSHA programs or standards. OSHA has broad workplace safety responsibilities which pertain to physical hazards such as electromagnetic radiation, temperature, noise and vibration; ergonomic hazards such as repeated motions or heavy lifting; and biological hazards including bacterial and viral pathogens, in addition to the chemical hazards that are the primary subject of this section. The Act also established the National Institute for Occupational Safety and Health (NIOSH) within the DHHS. NIOSH is authorized to develop and establish recommended occupational safety and health standards by conducting research and experimental programs for the development of new and improved occupational safety and health standards.

### **Exposure limits**

OSHA limits occupational exposure to hazardous chemicals by establishing exposure limits and directing changes in employer work processes or equipment, or when that is not feasible requiring personal protective equipment. Unlike drugs, biologics, pesticides or food additives, industry does not need to provide evidence of safety, or obtain an approval prior to manufacturing and marketing new products which may entail occupational exposure to hazardous chemicals. Thus, exposure limits may be adopted after the chemical has entered the occupational environment. The Act specifies that for regulating toxic chemicals OSHA must adopt standards which most adequately assure, to the extent feasible, on the basis of the best available evidence, that no employee will suffer material impairment of health or physical capability. Thus, some consideration must be given to the issue of technological achievability for industry in promulgating standards.

OSHA standards are called permissible exposure limits (PELs). They are legal standards that may not be exceeded. PELs have been controversial and susceptible to successful legal challenges. Thus, many chemicals do not have PELs. In the absence of OSHA PELs employers utilize recommended occupational exposure limits established by acknowledged sources in order to meet OSHA's requirements for worker safety. NIOSH has recommended exposure limits (RELs). The American Conference of Governmental Industrial Hygienists Threshold Limit Values (TLVs) for Chemical Substances and Physical Agents in the Work Environment are very widely utilized ([National Oceanographic and Atmospheric Administration, 1998](#)). TLVs refer to airborne concentrations of substances and represent conditions under which it is believed that nearly all workers may be repeatedly exposed day after day without adverse health effects. There are three categories of occupational exposure limits:

- 1 *Time-weighted average (TWA)*: The TWA concentration for a conventional 8-h workday and 40-h workweek, to which it is believed that nearly all workers may be repeatedly exposed, day after day, without adverse effect.
- 2 *Short-term exposure limit (STEL)*: The concentration to which it is believed that workers can be exposed for a short period of time without suffering from irritation, tissue damage or narcosis, it is not a separate exposure limit but supplemental to the TWA and limited to a 15-min TWA.
- 3 *Ceiling*: The concentration that should not be exceeded during any part of the working exposure ([The American Conference of Governmental Industrial Hygienists, 2005](#)).

The OSHA Hazard Communication Standard (29 CFR 1910.1200), also called the Right-to-Know Law, ensures that the hazards of all chemicals are evaluated and the information concerning their hazards is transmitted to employers and employees. It requires chemical manufacturers and importers to obtain or develop a material safety data sheet (MSDS) for each hazardous chemical they produce or import. Distributors are responsible to insure that their customers are provided a copy of these MSDSs. An MSDS must contain information describing the physical and chemical properties, health hazards, routes of exposure, precautions for safe handling and use, emergency procedures and control measures. The MSDS has become a well-established document for disseminating health and safety information about chemical products ([Occupational Safety and Health Administration, Technical Center Brochure, 2005b](#)). The NIOSH Pocket Guide to Chemical Hazards is another useful document for health and safety information

about hazardous chemicals ([National Institute for Occupational Safety and Health, 1997](#)).

## SECTION VI. STATUTES, REGULATIONS AND GUIDELINES

The three federal agencies (FDA, EPA and USDA), which are responsible for regulating animal health products, all maintain surveillance programs for monitoring products after they are marketed. The laws, which provide regulatory authority, and thus the regulatory goals, regulations and approach by these three agencies, are different. By extension, the pharmacovigilance programs utilized for monitoring the safety and effectiveness of marketed animal medicinal products are different. Thus, they have some common and some different statutes, regulations and guidelines.

### Notice and comment rulemaking procedures and access to agency records

Statutes are legislation passed by Congress and signed by the President. They are the laws that provide the authority for regulatory agencies to operate. Regulations are basic tools for achieving a regulatory agency's goals, such as consumer or environmental protection. Regulations inform the affected industries and the public of statutory requirements and the agency's procedures. Regulations interpret the law and spell out the details needed to implement the general provisions in the statutes. Regulations also describe the product approval process for many individual products or set forth required standards of product composition or performance.

Change is constant and agencies must issue new regulations and amend or revoke old ones. All regulations must be authorized by statute, and the rulemaking procedures must conform to requirements of the Administrative Procedures Act which applies to all executive branch departments and agencies of the federal government. The administrative process for establishing regulations used is referred to as "Notice and Comment Rulemaking."

New and amended regulations and other notices are disseminated by publication in the [Federal Register](#) which is issued daily by the Federal Government ([Federal Register](#)). Regulations are codified in the [Code of Federal Regulations](#) (CFR) which is updated annually. Preparing and issuing a regulation is a complex and lengthy process because agencies must be sure any new rule is needed and well conceived. Stakeholders such

as affected industry and consumers generally must be given an opportunity to participate through the notice and comment rulemaking process. Ordinarily, this is done by publishing in the [Federal Register](#) a notice of proposed rulemaking and inviting comments, within a specified time frame, typically 60 or 90 days. In appropriate cases public hearings are also held. The written and public hearing comments then must be reasonably responded to before issuing a final regulation. After reviewing all comments an agency publishes the final rule in the [Federal Register](#) and announces the effective date of the regulation. The regulation then becomes part of the CFR.

Guidelines establish practices of general applicability and do not include decisions or advice on specific situations. Guidelines are not legal requirements, but may be relied on with the assurance that it is an acceptable procedure or standard. A guideline represents the formal position of the agency on a matter and obligates the agency to follow it until it is amended or revoked. The guideline development process is similar to the regulation publication process. Draft guideline availability and request for comment is typically announced in the [Federal Register](#) and comments are considered when developing final guidelines.

### Freedom of information and public access to regulatory agency records

The Freedom of Information Act (FOIA) went into effect in 1967. It provides access to information in government files and requires that each government agency publishes descriptions of its operations and procedures. Each agency must also make available opinions, orders and statements of public policy that affect the public. Any person can obtain information through an FOIA request. Certain types of information are not available under FOIA. For example, national security-related trade secrets and commercial information and personnel and medical files are exempt. Agencies are given a certain period of time to process requests, typically within 20 days of receipt. The key to obtaining the desired information is to make the request sufficiently detailed and specific to allow identification of the record(s). Information that would be important to veterinarians would include adverse reaction information for animal drugs, regulatory letters written by the agency, Compliance Policy Guidelines (to direct FDA field staff in enforcement activities) and guidelines implementing regulations. Fortunately most of this information is now available on federal agency Internet home pages. For instance, Warning Letters to manufacturers for violations of Good Manufacturing Practices, and livestock producers or veterinarians involved in causing violative drug



residues may be viewed on FDA's FOIA site. Summary information on reports of adverse drug reactions is available on FDA CVM's home page.

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# Regulatory aspects for the drugs and chemicals used in food-producing animals in the European Union

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## INTRODUCTION

Drugs and chemicals are essential for the production of animals intended for food. They are used orally or systemically to treat animals or prevent disease and also to promote growth and feed efficiency. The European Union (EU) has already banned antibiotics and growth promoters such as avoparcin, ardacin, zinc bacitracin, virginiamycin, tylosin phosphate, spiramycin, monensin sodium, salinomycin sodium, avilamycin and flavophospholipol added to animal feed due to the fact that the use of these substances could lead to the selection of resistant strains in animals and this form of antibiotic resistance can present problems for antibiotics used in human therapy (Anadón and Martínez-Larrañaga, 1999; Anadón, 2006). Individual animals may be treated (i.e., individual injection or by oral gavage), but it is often more efficient to treat entire groups by mass application by drinking water or feed. Certain mass-medication procedures, called metaphylaxis (a form of prophylaxis), try to treat sick animal outbreaks while medicating others in the group to prevent disease. It is a continuing concern of consumers that various drugs and chemicals could be found at above maximum residue limits (MRLs) in food. The possible effect of such drugs and chemicals on our health and safety depends on the level of exposure to animals, in particular through the foods we eat. Foods containing residues of any of pharmacologically active substances (drugs and biocides) or other undesired

substances (chemicals) above the MRLs could potentially constitute a public health hazard and could create problems in international trade, in particular the substances that have no acceptable daily intake (ADI) and no international MRLs, or their MRLs do not cover species and uses which were considered minor by the sponsors of the data. The establishment of the MRL represents one of the several standard options for risk managers to limit the presence of unwanted substances.

In the EU, the major action was the introduction of requirements for MRLs for residues of veterinary drugs in food of animal origin. Residues are the traces of the drug and its metabolites which remain in the animal and its edible products after treatment with veterinary medicinal products. Depending on the nature of the chemicals and its metabolites they may pose hazards with concurrent risks to consumers when the concentrations are above a safe limit. For instance, certain substances having a thyrostatic, estrogenic, androgenic or gestagenic action, that they leave residues in meat and other foodstuffs of animal origin, may be dangerous to consumers and may also affect the quality of foodstuffs of animal origin. The concern for hormonal residues focuses on chronic exposure to low doses rather than an acute exposure to high doses. In summary, the hazards may be toxicological, pharmacological or microbiological in nature.

The detection of low levels of residues of certain prohibited veterinary drugs in imported animal products by laboratories in the EU at the end of 2001 and during

the first months of 2002 triggered a discussion within the *Codex Alimentarius* Commission whether and how the problems causing such events could be addressed. Several control laboratories in member countries of the EU detected trace amounts of chloramphenicol and nitrofurans in imported animal products (e.g., shrimps, chicken). These findings were triggered mainly by improvements of analytical methods which significantly lowered the levels of detection for residues of these drugs. The question to study is what supranational rules apply if an importing country detects residues of a veterinary drug of which the presence in a food product is not authorized or the use of the drug is not authorized by its own legislation. The relevant international framework which needs to be studied in order to answer this question is provided by treaties and agreements of the World Trade Organization (WTO) and the texts adopted by the *Codex Alimentarius* Commission (CAC) (Lützow, 2004).

The World Trade Organization started to work in 1995, its main objective being the facilitation and promotion of international trade. The main function of WTO is to serve as a monitoring forum for the implementation of a number of treaties which were agreed during the so-called Uruguay round (1986–94) and for settling of trade disputes subject to the rules laid down in those treaties. The more specific principles applied by WTO when developing rules and treaties for trade between her members are: countries may not discriminate trade partners; national and foreign companies shall be treated equally; and trade restrictions are justified for the health protection of humans, animals and plants.

The control of residues of veterinary drugs in animal products intended for human consumption is an issue related to human health which falls as such under the purview of the Agreement of Sanitary and Phytosanitary Measures (SPS). Under the SPS, WTO members can adopt SPS measures that are “necessary for the protection of human and animal health” (Lützow, 2004).

There are a number of veterinary drugs for which Codex has not adopted an MRL. An ADI/MRL may not have been established because the veterinary drug may not have been evaluated, the toxicological data did not support an ADI, the residue data were insufficient, a suitable validated analytical method was not identified or good agricultural practices would result in exceeding the MRL. In the absence of ADI/MRL, national authorities commonly resort to zero tolerance regulatory approaches (where no detectable residue of the veterinary drug in the food is acceptable), with the prevalence of residues of concern potentially changing as analytical method detection capabilities improve.

## VETERINARY MEDICINAL PRODUCTS LEGISLATION

Veterinary medicinal products legislation is regulated in the EU by Directive 2001/82/EC (EC, 2001). In the EU legislative framework for medicines, there are a large number of directives and regulations regarding the issuing of marketing authorizations and the economic regulation of the pharmaceutical market that need to be applied and implemented effectively.

The body of knowledge relating to this implementation is constantly evolving as the EU scientific committees and their working parties develop guidelines and gain experience in new therapies and new technologies.

### Marketing authorization procedures

Marketing authorization applications, which are to be submitted in either a national or Community procedure (i.e., to competent authorities of the Member States and the European Medicines Agency (EMA)), consist of administrative information and the necessary documentation to demonstrate the quality, safety and efficacy of the veterinary medicinal product. This applies to non-immunological and immunological veterinary medicinal products.

A veterinary medicinal product may only be placed on the market in the European Economic Area (EEA) when a marketing authorization has been issued by the competent authority of a member state (or EEA country) for its own territory (national authorization) or when an authorization has been granted in accordance with Regulation (EC) No. 726/2004 (EC, 2004a) for the entire Community (a Community authorization). The marketing authorization holder must be established within the EEA.

The authorization procedures have developed over the years to become very complex with four different routes of application: the centralized or community procedure, the decentralized (DCP), the mutual recognition (MRP) and the national procedure requiring a high level of bureaucracy both for industry and regulators without gain of increased quality, safety and efficacy.

#### Centralized or community procedure

The EMA is the EU body responsible for the scientific evaluation and monitoring of medicines within the “centralized procedure,” a procedure that allows a marketing authorization (license) for a medicine to be obtained in all EU countries simultaneously, on the basis of a single application. For veterinary medicinal products which fall within the mandatory scope of the centralized procedure in accordance with the Annex to Regulation (EC) No. 726/2004

(EC, 2004a), the application is submitted to the EMA. An application shall likewise be submitted to the EMA for veterinary medicinal products which fall within the optional scope of the centralized procedure in accordance with Article 3(2) and 3(3) of Regulation (EC) No. 726/2004 (EC, 2004) where the applicant wishes to obtain a Community marketing authorization. In the “centralized” procedure, the EMA-CVMP is responsible for conducting the initial assessment of veterinary medicines for which an EU-wide marketing authorization is sought. The EMA-CVMP is also responsible for several post-authorization and maintenance activities, including the assessment of any modifications or extensions (“variations”) to an existing marketing authorization.

Following the scientific evaluation and upon receipt of the opinion, the European Commission drafts a Decision on a Community marketing authorization and, after consulting the Standing Committee for Veterinary Medicinal Products, grants a marketing authorization.

#### *Decentralized procedure and mutual recognition procedure*

Evaluation of the operation of marketing authorization procedures has revealed the need to revise the mutual-recognition procedure in order to improve the opportunities for cooperation between Member States. Therefore, Directive 2004/28/EC (EC, 2004b) has introduced the decentralized procedure and the coordination group, which is responsible to settle any disagreements arising from the decentralized and mutual recognition procedures. Both the decentralized and the mutual recognition procedures are based on the recognition by national competent authorities of a first assessment performed by the authorities of one member state. To allow operation of the system, applicants for marketing authorization are obliged to include in their applications copies of any authorization previously obtained in other Member States as well as a list of those Member States in which an application for authorization is under examination (Article 12(3)(n) of Directive 2001/82/EC (EC, 2001)).

In the “mutual-recognition” and “decentralized” procedures, the CVMP arbitrates in cases where there is a disagreement between Member States concerning the marketing authorization of a particular veterinary medicine (“arbitration procedure”). The CVMP also acts in referral cases, initiated when there are concerns relating to the protection of public health or where other Community interests are at stake (“Community referral procedure”).

#### *Decentralized procedure*

The decentralized procedure is the most common route used and takes place in two stages – licensing in

one Member State (“national procedure”) followed by approval in other Member States (“mutual recognition”). In this “national procedure,” regulatory assessors will examine the product’s quality, safety and efficacy and, if content, will advise the competent authority to issue an authorization. Once a marketing authorization has been given in one member state, gaining EU-wide authorization is then possible on the basis of “mutual recognition” whereby the second and subsequent countries’ regulatory authorities should, subject to certain conditions, accept the first country’s “national procedure.” The entire process is known as the decentralized procedure.

For veterinary medicinal products not falling within the mandatory scope of the centralized procedure, the applicant may request one or more concerned member state(s) to approve a draft assessment report, summary of product characteristics, labeling and package leaflet as proposed by the chosen reference member state. An application is submitted to the competent authorities of the reference member state and the concerned member state(s), together with the information and particulars referred to in Articles 12–14 of Directive 2001/82/EC (EC, 2001). The applicant must give an assurance that the dossier, including the proposed summary of product characteristics, labeling and package leaflet, is identical as submitted in all Member States concerned (reference member state and concerned member state). Differences in proposed prescription status and names of the veterinary medicinal product are acceptable, in line with national rules in force.

At the end of the decentralized procedure with a positive agreement, a national marketing authorization will be issued in the reference member state and the concerned member state. The harmonization is maintained through the procedures of Regulation (EC) No. 1084/2003 (EC, 2003) for the examination of variations and the use of the decentralized and mutual recognition procedures for extensions.

#### *Mutual recognition procedure*

This procedure is based on the mutual recognition by concerned member state(s) of a national marketing authorization granted by the reference member state. The concerned member state refers to the reference member state that issued the national marketing authorization on which the mutual recognition procedure is based. At the end of the mutual recognition procedure, a national marketing authorization will be issued in the concerned member state(s). The harmonization is maintained through the procedures of Regulation (EC) No. 1084/2003 (EC, 2003) for the examination of variations and the use of the decentralized and mutual recognition procedures for extensions and renewals.



Independent national procedures

Independent national procedures will continue, but are strictly limited from January 1, 1998 to the initial phase of mutual recognition (granting of the marketing authorization by the reference member state) and to veterinary medicinal products which are not to be authorized in more than one member state.

In addition, as provided for in Article 34(2) of Directive 2001/82/EC (EC, 2001), harmonization of authorizations for veterinary medicinal products authorized in the Community is to be promoted via a coordinated approach for referring veterinary medicinal products, for which divergent decisions have been adopted, to the EMA and the CVMP.

Independent national procedures can also be used for extensions of authorized veterinary medicinal products as far as no *a priori* harmonization has been achieved for the initial marketing authorization.

The safety evaluation for veterinary medicinal product and its residues is based on a package listed in Part 3 of the Commission Directive 2009/9/EC (EC, 2009a) (Table 10.1).

HUMAN HEALTH RISKS FROM DRUG RESIDUES IN FOODS

Edible or target tissues containing veterinary drug residues can pose risk to human health, including direct toxic effects, allergic reactions and increases in bacterial resistance to common antibiotics.

The toxicity of drugs is an inherent part of all uses of medication, and there are differences from one animal or human to another, especially in allergic reactions. Residues of drugs or their metabolites in food products from treated food animals are major considerations in the safety of drugs approved for use in food animals. In Europe, EMA or Member States approve of drug dosages, routes of administration, durations of treatment and withdrawal times, and MRLs are designed to ensure

the safety of foods derived from treated animals. EU regulations have effectively prevented allergenic, toxic and potential carcinogenic drug residues from entering the food supply.

Any adverse reaction is likely to occur due to acute rather than long-term chronic effects.

Acute toxicity effects

For toxicology testing, the sponsor should perform acute and chronic dosing studies. For example, the acute impact of antibiotics is not directly examined in the toxicological studies required in the pre-approval process for veterinary drugs, since the primary concern has focused on chronic effects (i.e., carcinogenesis). The acute studies evaluate animals for problems such as allergies. Acute effects of food-borne drug residues on human health have also been described. These acute effects are defined as those that occur or develop rapidly after single administration of chemical substances. Some examples are hypersensitivity and pharmacological and teratogenic effects.

Hypersensitivity

Meat and other dietary products from treated food animals may contain residues of many antimicrobials or haptenized macromolecules. For small molecules to become immunologic, they must be able to form covalent bonds with macromolecules (i.e., proteins or polysaccharides and polynucleotides). Allergic reactions can be caused by beta-lactam drugs (penicillins and cephalosporins), aminoglycosides, sulfonamides and in a few cases nitrofurans, and tetracyclines. Adverse human reactions are manifested as severe swelling of the skin, serum sickness and shock; less serious reactions such as skin rashes, asthma and fever have been described in sensitive humans due to prior treatment by antibiotics either with the same drug or with one closely related. Drug sensitization can be acquired by antimicrobial

TABLE 10.1 Safety and residue tests in respect of testing of veterinary medicinal products

3A. Safety tests	3B. Residue tests
Precise identification of the product and of its active substance(s); Pharmacology (Pharmacodynamics, Pharmacokinetics); Toxicology (Single-dose toxicity, Repeat-dose toxicity, Tolerance in the target species, Reproductive toxicity including developmental toxicity ( <i>Study of the effects on reproduction, Study of developmental toxicity</i> )), Genotoxicity, Carcinogenicity, Exceptions; Other requirements (Special studies, Microbiological properties of residues ( <i>Potential effects on the human gut flora, Potential effects on the microorganisms used for industrial food processing</i> )), Observations in humans; Development of resistance; User safety; Environmental risk assessment (Environmental risk assessment of veterinary medicinal products not containing or consisting of genetically modified organisms, Environmental risk assessment for veterinary medicinal products containing or consisting of genetically modified organisms).	Introduction; Metabolism and residue kinetics (Pharmacokinetics (absorption, distribution, metabolism, excretion); Depletion of residues); Residue analytical method

drugs, their metabolites or degradation products. However, cases of proven allergy to such substances in food are extremely rare, based on clinical and laboratory experience of an immunological reaction, whereas there are less well-substantiated reports blaming antibiotics in up to 50% of cases of chronic urticarial (Dayan, 1993). Reportedly, both epidemiologic and experimental data indicate that levels of penicillin as low as 5 to 10 IU are sufficient to produce an allergic reaction in previously sensitized individuals (Sundlof, 1994). Adverse human reactions occur at very low doses, and it is highly probable that allergic individuals to the drug previously enumerated, when exposed via the food, could suffer an allergic reaction. Probably the reason that few cases are documented is that many cases might be masked with other health conditions, especially in elderly populations, as well as problems with underestimation and underreporting.

Two veterinary drugs may be toxic to humans when used therapeutically. Both are used in veterinary medicine causing adverse reactions by mechanisms involving hypersensitivity and where there is no clear relationship with dose. The first one is chloramphenicol, which may produce two types of bone marrow damage: a reversible dose-related interference with iron metabolism and an irreversible idiosyncratic form of an often fatal aplastic anemia. The mechanism for pathogenesis of aplastic anemia is known, but it may be involved in reduction of the nitro-group to produce toxic metabolites. The reversible form is likely to occur with high doses, a prolonged duration of treatment and in patients with liver disease. Irreversible idiosyncratic aplastic anemia occurs in <1:25,000 patients given chloramphenicol. The onset may be delayed until after therapy has been discontinued. The "gray baby syndrome", which is often fatal, occurs in newborns related to the inability of the immature liver to metabolize chloramphenicol and manifests with standard doses. The second drug is levamisol, which is known to cause reversible agranulocytosis in humans, in which case the mechanism is unknown. The levamisol is widely used as an anthelmintic in animals and the possible occurrence of its residues in animal tissues has caused concern (Woodward, 1991).

### Pharmacological effects

Pharmacological effects may help in the understanding of toxicological phenomena and toxicological effects may result from exaggerated pharmacological responses. These adverse pharmacological effects are generally regarded as acute effects. Similarly, the pharmacological effects of some drugs can be important (e.g., clenbuterol, isoxuprine). This was illustrated by incidents where the  $\beta$ -agonist action of the drug clenbuterol resulted in adverse effects in consumers of liver containing residues of the drug. NOELs can be

determined for pharmacological effects and then used in the calculation of the ADI. It is known that there are two major classes of adrenergic receptors,  $\alpha$  and  $\beta$ , based on the relative effectiveness of several adrenergic compounds to produce a variety of actions in different body tissues. There are two  $\beta$  receptor subtypes,  $\beta_1$  and  $\beta_2$  based on the relative potency of a series of compounds with structural similarities to epinephrine and norepinephrine on physiological processes, including cardiac stimulation and bronchodilation. The  $\beta_2$  receptors predominate in smooth muscles and blood vessels whereas  $\beta_1$  receptors predominate on the heart. Metabolic effects such as glycogenolysis in skeletal muscle or liver and lipolysis are mediated primarily through  $\beta_2$  and  $\beta_3$  receptors are expressed predominantly in adipose tissue.

It is known that the  $\beta$ -agonist clenbuterol has been used illegally as growth promoters in farm animals as a non-steroidal anabolic and metabolism accelerator. Clenbuterol was the first of the synthetic phenethanolamine compounds with demonstrated ability to increase lean and decrease fat in food animals. In addition to their therapeutic effects,  $\beta_2$ -agonists are effective also as "repartitioning agents." As a growth promoter clenbuterol was the first of the synthetic phenethanolamine compounds with demonstrated ability to increase lean and decrease fat in food animals. The use of clenbuterol to enhance lean tissue growth requires a dose that is 10 times higher than the normal therapeutic dose of 0.8  $\mu\text{g}/\text{kg}$  body weight. Factors affecting response to  $\beta$ -adrenergic agonist include level of dietary protein, length of treatment, genotype and sex. Clenbuterol, a selective  $\beta_2$ -agonist, is used as a bronchodilator in human and veterinary medicine and it is available as 0.01 mg and 0.02 mg tablets as well as respiratory solution. In humans, it is used at the recommended oral dosage of 0.02–0.03 mg twice daily. The eye, vitreous humor and retina have been suggested as tissues for residue screening. More recently, accumulation of clenbuterol in hair has been demonstrated and suggested as a sensitive indicator of  $\beta$ -adrenergic agonist residues. In animals in the EU is listed in Table I of the Annex of Commission Regulation (EU) No. 37/2010 (EU, 2010) as allowed substance (Table 10.2).

According to the Directive 2008/97/EC (EC, 2008a) the  $\beta$ -agonists can be used to induce tocolysis (relax the uterus) in cows when calving as well as to treat respiratory problems, navicular disease and laminitis and to induce tocolysis in *equidae*.

In contrast to other  $\beta_2$ -agonists, clenbuterol is well absorbed after ingestion (bioavailability 70–80%). Peak serum levels occur within 3 hours and the elimination half-life was 25–39 hours. At larger doses, it also shows  $\beta_1$ -receptor-mediated (cardiac) effects.

The anabolic effect of clenbuterol, and other long-acting  $\beta_2$ -agonists, appears to be mediated through the  $\beta_2$ -receptors in skeletal muscle. Through its thermogenic

TABLE 10.2 Clenbuterol and its classification regarding maximum residue limits (MRL)

Pharmacologically active substance	Marker residue	Animal species	MRL	Target tissues	Therapeutic classification
Clenbuterol hydrochloride	Clenbuterol	Bovine, <i>Equidae</i>	0.1 µg/kg 0.5 µg/kg 0.5 µg/kg	Muscle Liver Kidney	Agents acting on the nervous system/ Agents acting on the autonomic nervous system
		Bovine	0.05 µg/kg	Milk	

properties, clenbuterol increases energy expenditure and hence reduces muscle glycogen and body fat deposition. In farm animals, clenbuterol accumulates in the lung, liver and kidney, and to a lesser extent in the muscle. Residues of  $\beta$ -agonists in animal tissues used for food may result in serious human health risk.

Clenbuterol has been implicated in several outbreaks of food-borne poisoning follow the consumption of liver, meat and lung of bovine and lung and liver of pig. In 1990, a poisoning incident was reported in Spain where 135 people became ill after consuming beef liver containing clenbuterol residues (Martinez-Navarro, 1990). A second episode of clenbuterol food poisoning was reported one year later in France and affected 22 people, who consumed veal liver, in eight different families in two different sites. The patients suffered tachycardia and tremors for 2 or 3 days. The infective dose is calculated as 1–2 µg/kg/day (Pulse *et al.*, 1991). In 1992, a further episode of clenbuterol poisoning appeared in Spain after ingestion of veal liver. Analysis of liver indicated concentrations of clenbuterol in the range of 160–500 µg/kg. Consumption of 100 g of liver would already exceed the pharmacological effect level of clenbuterol of 5 µg/person. Clenbuterol was detected in 47 urine samples in amounts ranging from 11 to 486 ppm. Other episodes appeared in Hong Kong (1997–98) after the consumption of pig lung (16 people) or pig liver (one people), and in China (2009) 70 people fell ill after eating pork products contaminated with clenbuterol. The common clinical symptoms in food-borne clenbuterol poisoning consisted of muscle tremors, heart palpitation/sinus tachycardia, nervousness, general myalgia, fever, nausea/vomiting, headache, dizziness/vertigo and chills. The latency period varied between 10 minutes and 6 hours, and the duration of symptoms between 90 minutes to 6 days (mostly lasting less than 3 days). Some patients also presented with weakness or confusion. Others developed transient, mild hypokalemia. Electrocardiograms for some patients revealed sinus tachycardia of 120–150 beats/min with ventricular and supraventricular ectopics. Patients with underlying cardiac disease are generally expected to be more susceptible to the cardiac effects of clenbuterol. There were no reported deaths.

One of the side effects of animal treatment with  $\beta$ -adrenergic agonists is increased heart rate. This effect may be either a direct or indirect effect of treatment. The acute toxic effects observed in animals include muscle tremors, tachycardia and nervousness. In addition, treated animals show a higher incidence and severity of hoof lesions. Furthermore, specific histological changes have been observed in treated animals, in particular the formation of vacuoles of epithelial cells in the prostate and Bartholin's gland of male and female calves, respectively. The levels of clenbuterol in the edible tissues are expected to be at the highest.

#### Teratogenic effects

Another acute toxic effect that may occur as a result of exposure to violative animal drug residues is a teratogenic effect. Teratogens are active at very low doses and even brief exposure during a critical period of development can result in a deformation which lasts a lifetime; although the hazard associated with teratogens is severe, the possibility of the event occurring is infrequent. Therefore, the changes to a woman at the critical stage of pregnancy coming in contact with a violative residue are very low (Paige *et al.*, 1997). Other effects caused by a single exposure can be a haemotoxicity, neurotoxicity and endocrine effects.

#### Long-term chronic effects (carcinogenesis)

The long-term chronic toxicity studies identify if problems such as cancer are associated with a specific drug. This study will determine a dose that does not create any health problems. Most residues of veterinary medicines or chemicals occur in food at low concentrations, therefore they rarely pose a chronic or long-term health hazard to consumers. Furthermore those effects as a result of exposure to drug or chemical residues in food are particularly difficult to detect and are certainly under-ascertained as well as under-reported. Carcinogenic studies are a cornerstone of toxicological assessment; however, carcinogenicity studies are rarely used to establish a NOAEL. Special problems emerge where the substance

in a genotoxic carcinogen or a mutagen, due to no threshold, can in theory be identified for it. A substance may not have ADI and MRL for a variety of reasons, in particular when the important pivotal studies were missing or were of insufficient quality and the elaboration procedure had to be discontinued because the sponsor was not prepared to conduct further work. An example can be the carcinogenicity studies. No allocation of an ADI and a no recommendation of MRL mean that safety concerns as a result of the substance were negatively evaluated by a regulatory competent body.

### *Antimicrobial effects*

Antimicrobial drug residues in contaminated foodstuffs can lead to direct toxic effects. The impact may range from sensitizing reactions to drug-inducing organ damage, or may be both. Moreover, to direct toxic effects, many trace amounts of antimicrobial drugs may have the potential to perturb the human gut flora or to disrupt the barrier effect which it exerts, thus permitting the ingress of potentially pathogenic bacteria into the gastrointestinal tract. Antimicrobial residues in food constitute a variety of health hazards to humans which depend on the frequency and degree of residue exposure. The two main risks related to antimicrobials are hypersensitivity reactions, which appear in allergic individuals, and the acquisition by pathogenic microorganisms of resistance to certain antibiotics. These effects may be studied using a variety of experimental models, and they can be taken into account in the calculation of the ADI value.

The standard human food safety assessment for new animal drugs accurately determines the safe concentration for traditional toxicological endpoints as mentioned above. However, the impact of low levels of antibiotics on the intestinal microflora is not directly examined in these toxicological studies. Therapeutic doses of antibiotics can cause adverse effects on the intestinal microfloral ecology (i.e., disruption of the intestinal microflora, or effects on the metabolic activity of intestinal microflora). The adverse effects of antimicrobials are a concern because of the important role that the intestinal microflora plays in maintaining an individual's health. Also perturbation of the intestinal microflora may compromise the effectiveness of other drug therapies and thereby adversely affect public health. Most studies of antimicrobial drugs and their effects on the human intestinal microflora were performed with therapeutic levels of antibiotics. In contrast to the well-documented negative effects of therapeutic dose of antibiotics, the effect of low levels (i.e., ppb or ppm) of antibiotics on perturbing the intestinal microflora is not well defined. It is possible that low doses of antimicrobials agents, such as those found as residues in foods, could alter intestinal enzyme activity and have an effect on certain hormones

and drugs, since in most cases the lowest doses at which the perturbations in the intestinal microflora occur have not been determined. In order to ensure human food safety FDA's CVM considered data gathered from a large number of compounds and determined that the maximum safe concentration for antimicrobial products is 1 ppm in a total diet of 1.5 kg. This equals a maximum antibiotic dose of 1.5 mg/day from consuming residues in food. This level of an antimicrobial residue in food should produce no effects on the intestinal microflora (Paige *et al.*, 1997).

Antimicrobial feed additives have been used in animal production for many decades because of their favorable economic effects in livestock production. Added in low doses to the feed of farm animals, they improved growth and performance and hence were known as antimicrobial growth promoters. Due to the emergence of microbes resistant to antibiotics ("antimicrobial resistance") that are used to treat human and animal infections, the European Commission (EC) decided to phase out, and ultimately ban since January 1, 2006, the marketing and use of antibiotics as growth promoters in animal feed. Since then, the use of antibiotics is now only allowed on veterinary prescription for direct applications or as medicated feed. These restrictions are deemed necessary as antimicrobials may lead to the selection of resistant bacterial strains in animals that could be transferred to humans, by direct contact or via foodstuffs, and subsequently lead to an impairment of the efficacy of antibiotics used in therapy of human infectious diseases.

### *Hormonal effects*

Growth promoting hormones, more particularly steroids and xenobiotics, have been used (legally and illegally) to increase live-weight gain and feed efficiency in various species, more intensively in cattle and to lesser extent in ovine, swine, poultry and fish. Promoting steroid hormones can be divided into four groups of drugs: (1) the natural steroids (oestradiol-17 $\beta$ , progesterone, testosterone); (2) the xenobiotics (trenbolone acetate and zearanol); (3) the synthetic steroids (ethinylestradiol, methyltestosterone, chlormadinone acetate and others); and (4) the diethylstilbestrol and related substances (hexestrol, dienestrol).

The endogenous steroid hormones have a wide range of actions which affect almost all systems of the body. These hormones act by binding with high affinity and high specificity to protein receptors which are located in hormone-responsive tissues. The toxicity or adverse effects of the steroid hormones in humans are apparent even at physiological concentrations. A physiological level of testosterone contributes to acne, baldness, prostatic disease and prostate cancer, cardiovascular disease and peptic ulcers. Physiological concentrations



of estrogens, and to a lesser extent progesterone, have been implicated in premenstrual tension, dysmenorrhea and some cancers of the reproduction system including breast cancer, uterine and cervical cancer (Taylor, 1983). It has been described that the estrogen causes induction of DNA adduct formation. The endogenous hormones are regulated as promoters rather than primary inducers of cancer in hormonally sensitive tissues.

The natural sex steroid hormones estradiol, testosterone and progesterone may be in use illegally for growth promotion.

Diethylstilbestrol (DES) is perhaps the most widely known of the estrogenic growth promoting agents. It is structurally different from the endogenous estrogens in that it does not possess the fused steroid ring structure but does possess two phenolic hydroxyl groups which give DES a special orientation similar to estradiol. The relative binding affinity of DES for the estrogen receptor is 141% (versus 100% for estradiol), but DES is 10–20 times more active than estradiol by the oral route. DES is also metabolized to radical or quinone intermediates which react with cellular macromolecules, including DNA (Metzler, 1984). DES is carcinogenic in humans and in laboratory animals. DES was first used therapeutically in pregnant women in the U.S. to prevent threatened abortion, and in the early 1970s the link was discovered between clear adenocarcinoma in women and their exposure to DES *in utero* (Herbst *et al.*, 1971; Meyers, 1983). Furthermore, it has been shown that DES had other effects on daughters and sons of mothers treated with DES being the most common vaginal adenosis and other gross abnormalities of the reproductive tract (Herbst, 1976).

According to Directive 2008/97/EC the following substances are prohibited: thyrostatic substances; stilbenes, stilbene derivatives, their salts and esters; and estradiol 17 $\beta$  and its ester-like derivatives (see Table 10.3).

The Opinion of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) (EC, 2002a) concluded that there is a substantial body of recent evidence suggesting that 17 $\beta$ -estradiol has to be considered as a complete carcinogen, as it exerts both tumor-initiating and tumor-promoting effects, and that the data currently available do not make it possible to give a quantitative estimate of the risk to human health. The mutagenic and genotoxic potential of 17 $\beta$ -estradiol is a consequence of metabolic activation to reactive quinones. The report concludes that 17 $\beta$ -estradiol is not essential in the production of food-producing animals because the use of the available alternatives (especially prostaglandins) by practicing veterinarians is already quite common in the Member States and that the complete prohibition of the use of 17 $\beta$ -estradiol for food-producing animals would have no, or only a negligible, impact on farming and animal welfare (EC, 2002a). Studies on the

TABLE 10.3 Substances not permitted in the EU

1. Substances having a hormonal (stilbenes, stilbene derivatives, their salts and esters, estradiol 17 $\beta$  and its ester-like derivatives) or thyrostatic action, and of  $\beta$ -agonists (with derogations) (Council Directive 96/22/EC) (Directive 2008/97/EC).
2. Pharmacologically active substances listed in Annex, Table 2 of Commission Regulation (EU) No. 37/2010 (Aristolochia spp. and preparations thereof, chloramphenicol, chloroform, chlorpromazine, colchicine, dapsone, dimetridazole, metronidazole, nitrofurans (including furazolidone), ronidazole).
3. Antibiotic growth promoters (avoparcin, ardacin, zinc bacitracin, virginiamycin, tylosin phosphate, spiramycin, monensin sodium, salinomycin sodium, avilamycin and flavophospholipol added to animal feed due to the fact that the use of these substances could lead to the selection of resistant strains in animal and this form of antibiotic resistance can present problems for antibiotics used in human therapy (Council Regulation (EC) No. 2821/98)). Growth factors (quinoxaline-N-dioxides such as carbadox and olaquindox presented a risk to consumers (possess genotoxic and/or carcinogenic properties), to operators or to the animal itself (impairment of adrenal cortex function in pigs) (Commission Regulation (EC) No. 2788/98)).
4. Pharmacologically active substances not listed in the Annex, Table 1 (allowed substances) of Commission Regulation (EU) No. 37/2010 (i.e., MRLs do not cover species and uses which were considered minor by the sponsors of the data).

metabolism of 17 $\beta$ -estradiol in bovine species indicate the formation of lipoidal esters, disposed particularly in body fat. These lipoidal esters show a high oral bioavailability in rodent experiments, thus, the consequence of their consumption needs to be considered in a risk assessment. It is known that experiments with heifers indicated a dose-dependent increase in residue levels of all hormones particularly at the implantation sites. Misplaced implants and repeated implanting, which seem to occur frequently, represent a considerable risk that highly contaminated meat could enter the food chain (EC, 2002a).

Zearanol and trenbolone have been tested for their mutagenic and genotoxic potential in various systems with different endpoints; both compounds exhibited only very weak effects (EC, 2002a).

The new high-technology product bovine somatotropin (BST), used for the enhancement of milk production in cattle, is also not authorized in the EU.

### Carry-over or cross-contamination of the feed of coccidiostats

Different studies have shown that an entire contamination-free production of premixes and compound feeds in existing multi-product plants is not possible in practice (Strauch, 2002, 2003). Practical experience indicates that in feed mills, residual quantities of medicated feedingstuffs may be retained at various points along

the production line and end up at the beginning of the production of another feed product, contaminating subsequent batches of meal as they are processed. This unavoidable carry-over or cross-contamination may occur at all stages of production and processing of feed but also during storage and transport to feed localization (EFSA, 2007) although there are limited data on the amount and frequency of contamination of food with residues of coccidiostats resulting from feeding cross-contamination feedstuffs to food-producing animals (Dorne *et al.*, 2011).

Cross-contamination of feed batches can result in the exposure of non-target animals and induce adverse health effects in these animals due to a specific sensitivity of mammalian species as compared to poultry. Residue formation in edible tissues of non-target species may result in unexpected human exposure through the consumption of animal products (Dorne *et al.*, 2011). Feed additives, such as coccidiostats (polyether and non-polyether ionophores) for poultry, are marketed as premixes, intended to be incorporated into mixed feeds during the mixing and production process. The degree of carry-over depends on the technical facilities and procedures, as well as on product characteristics. For example, the physicochemical characteristics of feed additives can contribute to cross-contamination. The electrostatic properties of some drugs, particularly those in powder form, aggravate the problem, making it more difficult to clean the equipment between batches (Hurd, 1996). The feed additives and premixes also have an important influence on cross-contamination behavior, having the following properties of importance: adhesion strength – adhesion to walls – particle size and density (carrier, substance), and electrostatic properties. The cross-contamination decreases according to the product being less adhesive and electrostatic (EFSA, 2007).

The legal basis of this technological process is based on Regulation (EC) No. 183/2005 (EC, 2005) which replaced Council Directive No. (EC) 95/69 (EC, 95). Article 10 of Regulation No. (EC) 183/2005 (EC, 2006b) provides that feed business operators shall ensure that establishments under their control are approved by the competent authority in case these establishments are manufacturing and/or placing on the market coccidiostats and histomonostats, or premixtures containing coccidiostats and histomonostats.

Carry-over or cross-contamination has been evaluated (Strauch, 2002, 2003; Dorne *et al.*, 2011). Regular investigations have been performed with some coccidiostats, involving lasalocid and nicarbazin (McEvoy *et al.*, 2003; Noser *et al.*, 2006) and showed the persistence of these compounds in various feed batches produced after the intentional incorporation of a polyether ionophore coccidiostat into feed. The health risk to non-target species resulting from the consumption of cross-contaminated feed with coccidiostats at levels of 2, 5 or 10% was evaluated recently

by the European Food Safety Authority (EFSA) and a revision of the risk assessments performed can be found in the paper of Dorne *et al.* (2011). Overall, the toxic syndromes in non-target animal species are related to: (1) incidental consumption of fortified feeds (in most cases intended for chickens) by other animal species; (2) feed-mixing errors or ingestion of premix concentrates with unsafe amounts of ionophores; (3) off-label use, either accidental or intentional, have resulted in adverse reactions in adult poultry (laying hens), ostriches, ornamental and game birds, between others, and humans; and (4) drug interaction with other veterinary medicinal products (target and non-target animal species) (Dorne *et al.*, 2011).

Technical improvement can reduce this cross-contamination considerably and hence limits of cross-contamination can be defined per product to exclude any health risk for human and non-target animal species.

Recently, maximum tolerances for the presence of active substances contained in coccidiostats have been established in food of animal origin and in feeding-stuffs (Commission Regulation (EC) No. 124/2009 (EC, 2009b); Commission Directive 2009/8/EC (EC, 2009b)). The occurrence of unavoidable carry-over or cross-contamination of coccidiostats in non-target feed, even below maximum levels established, may result in the presence of residues of coccidiostats in food products of animal origin (i.e., in feed for which the use of coccidiostats or histomonostats are not authorized, such as feed intended for animal species or categories not provided for in the additive authorization). The maximum levels were set out for the coccidiostats lasalocid sodium, narasin, salinomycin sodium, monensin sodium, semduramicin, maduramicin, robenidine, decoquinate and halofuginone. However, other antimicrobials used as premixes for medicated feedingstuffs have not yet been evaluated for this purpose.

## Reference point for action

With the minimum required performance limits (MRPLs) the European Commission has proposed a temporary measure to address urgent problems related to prohibited or not authorized substances in the EU, in particular in relation to third country trade. Commission Decision 2002/657/EC introduced MRPLs intended to ensure harmonized implementation of Directive 96/23/EC (EC, 1997) for substances for which no permitted limits have been established. Reference point for action of MRPLs are defined as minimum content of an analyte in a sample, which at least has to be detected and confirmed. In contrast to MRLs for substances that may be used in food-producing animals in the EU, the MRPLs would be control tools based on expert advice on feasibility of controls, and other legitimate factors in risk

analysis. Consignments containing residues above the MRPLs would be rejected. If guarantees are provided, they may be returned to the sender.

The MRPLs laid down in Annex II to Commission Decision 2002/657/EC (EC, 2002b) are used as “reference points for action” irrespective of the matrix tested. The Commission Decision 2003/181/EC (EC, 2003) amended Decision 2002/657/EC (EC, 2002b), and established MRPLs for different antimicrobials (see Table 10.4). Where results of analytical tests on products: (1) are at or above the MRPLs, the consignment concerned shall be considered non-compliant with Community legislation and (2) are below the MRPLs, the products will not be prohibited from entering the food chain.

According to Article 1 of Regulation (EC) No. 470/2009 (EC, 2009a), the “reference point for action” is defined as the level of a residue of a pharmacologically active substance established for control reasons in the case of certain substances for which an MRL has not been laid down. The MRPL correspond to the average limit above which the detection of a substance or its residues can be construed as methodologically meaningful. Regulation (EC) No. 470/2009 (EC, 2009c) states that the setting of reference points for action should in no way serve as a pretext for condoning the illegal use of prohibited or non-authorized substances to treat food-producing animals. Therefore, any residues of those substances in food of animal origin should be considered undesirable. Regulation (EC) No. 470/2009 (EC, 2009c) highlighted that the reference points for action shall be reviewed regularly in the light of new scientific data relating to food safety and the outcome of the investigations and analytical tests, and technological progress. In conclusion, the EU would demand from its trading partners prohibition of the use of substances banned in the EU or to establish split systems and ask third countries to provide respective guarantees. In consequence detection of prohibited substances below the MRPL would still be documented and reported.

**TABLE 10.4** Minimum required performance limits (MRPLs) established for several antimicrobials

Substance and/or metabolite	Matrixes	MRPL
Chloramphenicol	Meat, eggs, milk, urine, aquaculture products and honey	0.3 µg/ml
Nitrofurantoines: furazolidone, furaltadone, nitrofurantoin, nitrofurazone	Poultry meat, aquaculture products	1 µg/ml for all

## BIOCIDAL SUBSTANCES USED IN ANIMAL HUSBANDRY

Biocidal substances used in animal husbandry are considered to be biocidal products used for the purposes of caring for and rearing food-producing animals, and to which food-producing animals are exposed during some stage of their lifetime. However, biocidal substances are used in many different situations and residues of biocidal substances may potentially enter the food chain as a result of a number of these uses (including exposure of plants to biocides, exposure of food-producing animals to biocides and contamination of food commodities with biocides). For instance, pesticide is defined as a plant protection product (Regulation (EC) No. 1107/2009) (EC, 2009a) and biocidal products as active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on harmful organism by chemical or biological means (Article 1(a) of Directive 98/8/EC (EC, 1998a)). Biocidal product types include those intended to control vertebrates; however, the actual use of such types might give rise to concern. Where a biocidal product identified as an insecticide, acaricide, rodenticide, avicide or molluscicide is authorized according to Directive 98/8/EC (EC, 1998) it is also subject to classification, packaging and labeling according to Council Directive 78/631/EEC (EEC, 1978).

Article 5(1)(b)(iii) of Directive 98/8/EC (EC, 1998a) specifies that for biocidal products/active substances that, as a result of their use, may lead to residues in food, Member States shall ensure that products are only authorized if these residues have no adverse effects on human health.

Article 10 of Regulation (EC) No. 470/2009 (EC, 2009a) provides for the setting of MRLs for pharmacologically active substances used in biocidal products in animal husbandry and specifies that the EMA is responsible for recommending MRLs for these substances.

Under the conditions of use, the biocidal products shall pose only a low risk to humans, animals and the environment. An active substance cannot be included in Annex 1A (list of active substances with requirements agreed at Community level for inclusion in biocidal products) if it is classified as carcinogenic, mutagenic, toxic for reproduction, sensitizing or is bioaccumulative and does not readily degrade. Inclusion of an active substance in Annexes I, IA or IB (list of basic substances with requirements agreed at Community level) shall, where appropriate, be subject to the following: (1) requirements on: (a) the minimum degree of purity of the active substance, (b) the nature and maximum content of certain

impurities, (c) product type in which it may be used, (d) manner and area of use, (e) designation of categories of users (e.g., industrial, professional or non-professional), (f) other particular conditions from the evaluation of the information which has been made available in the context of this Directive; (2) the establishment of the following: (a) acceptable operator exposure level (AOEL), if necessary, (b) where relevant, an acceptable daily intake for humans (ADI) and a maximum residue limit (MRL), (c) fate and behavior in the environment and impact on non-target organisms.

An exhaustive list of 23 product types (PT) can be found in Annex V of Directive 98/8/EC. Main group and product types which can be relevant for substances that may be used in animal husbandry are shown in Table 10.5 and therefore an LMR should be established.

## FEED ADDITIVES LEGISLATION

The new Regulation (EC) No. 1831/2003 (EC, 2003) sets out the rules for the authorization, use, monitoring, labeling and packaging of feed additives. This Regulation replaces Council Directive 70/524/EEC (EEC, 1970).

Directive 70/524/EEC (EEC, 1970) was fully reviewed in 2003 and Regulation (EC) No. 1831/2003 (EC, 2003) represented a major overhaul of the existing EU legislation on feed additives. The Regulation introduced many new aspects into the then existing legislation on feed additives, having been one of the first pieces of food safety legislation adopted following Regulation (EC) No. 178/2002 (EC, 2002b) by introducing the granting of time-limited authorizations valid for a period

of 10 years, the setting up of a Community Reference Laboratory for feed additives, the possibility of establishing MRL for certain additives which may result in residues when added to feed, and the possibility of laying down post-marketing monitoring programs at the time of authorization, as well as other provisions. The Regulation also kept coccidiostats and introduced histomonostats as a new category of feed additive, while establishing the phasing out of the use (and marketing) of the existing antibiotics as feed additives from January 1, 2006, taking into account the risks of selecting bacterial strains resistant to human or veterinary medicine drugs when using antimicrobials as growth promoters.

In 1997, the European Commission introduced the requirement to monitor the occurrence of resistance in animal bacteria associated with the use of antimicrobial feed additives and related substances (EC, 1997), suspending, at the same time, the use of avoparcin as a feed additive in January 1997 and ardacin in January 1998. This obligation was reconfirmed by Council Regulation (EC) No. 2821/98 (EC, 1998a) suspending in December 1998 the use of four other antibiotics (zinc bacitracin, virginiamycin, tylosin phosphate and spiramycin) that had been used as growth promoters in feed under the conditions that their use should be re-examined (Anadón and Martínez-Larrañaga, 1999). Subsequently, the European Commission supported a surveillance program, conducted by industry, to monitor antimicrobial resistance against feed additives in bacteria isolated from pigs and broiler chickens in the slaughterhouses in six European countries. However, as stated in the "White Paper on Food Additives" (adopted January 2000), the European Commission at that time already considered the prohibition or phase-out of antibiotics used as growth promoters within the EU, as part of a broader strategy to control and

TABLE 10.5 Biocides. Main groups and product types to be used in animal husbandry

Main group	Product type	Descriptions
1 Disinfectants and general biocidal products	3	Veterinary hygiene biocidal products: biocidal products used for veterinary hygiene purposes including products used in areas in which animals are housed, kept or transported.
1 Disinfectants and general biocidal products	4	Food and feed area disinfectants: products used for the disinfection of equipment, containers, consumption utensils, surfaces or pipework associated with the production, transport, storage or consumption of food, feed or drink (including drinking water) for humans and animals.
1 Disinfectants and general biocidal products	5	Drinking water disinfectants
3 Pest control	18	Insecticides, acaricides and products to control other arthropods: products used for the control of arthropods (e.g., insects, arachnids and crustaceans).
3 Pest control	19	Repellents and attractants: products used to control harmful organisms (invertebrates such as fleas, vertebrates such as birds), by repelling or attracting, including those that are used for human or veterinary hygiene either directly or indirectly.
4 Other biocidal products	20	Preservatives for food and feedstocks: products used for the preservation of food or feedstocks by the control of harmful organisms.



combat antibiotic resistance. With the aim of avoiding economic losses, in particular in the production of pigs and broiler chickens, and to avoid a significant increase in the use of antimicrobials under veterinary prescriptions, the EC supported the view that the phasing out of antimicrobial feed additives would be easier to pursue if other classes of growth promoting additives could be licensed. Subsequently, several microorganisms or probiotics and an organic acid product were authorized for use as growth promoters. Moreover, enzyme preparations, such as glucanases, xylanases, proteases and phytases, as well as prebiotics (fructo-oligosaccharides and related compounds), were granted marketing authorization.

The individual authorizations contain the characteristics of the products, the identification of the authorization holder, the maximum, minimum and/or recommended dosages, the animal categories in which they can be used, MRL and withdrawal periods where necessary, as well as specific labeling provisions and further conditions where necessary.

The FEEDAP Panel of EFSA carries out its work either in response to requests for scientific advice from risk managers or on its own initiative. Most commonly, and following specific authorization procedures, the European Commission asks EFSA to provide scientific advice and evaluate the safety and/or efficacy of a given substance in the context of its authorization for use in the EU. The scientific assessment performed by the Panel is based on Terms of Reference provided by the European Commission or other European bodies or determined by self-tasking. These guide the Panel approach to each question. Therefore, the authorization of an additive should be granted by the Commission

Article 5 of Regulation (EC) No. 1831/2003 (EC, 2003) states that a feed additive: (1) shall not have an adverse effect on animal health, human health or the environment, (2) shall not be presented in a manner which may mislead the user, and (3) shall not harm the consumer by impairing the distinctive features of animal products or mislead the consumer with regard to the distinctive features of animal product.

According to Regulation (EC) No. 1831/2003 a feed additive is a substance, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more specific functions that are enumerated in Article 5(3) of the Regulation (EC) No. 1831/2003 (EC, 2003): (1) favorably affect the characteristics of feed; (2) favorably affect the characteristics of animal products; (3) favorably affect the color of ornamental fish and birds; (4) satisfy the nutritional needs of animals; (5) favorably affect the environmental consequences of animal production; (6) favorably affect animal production, performance or welfare, particularly by affecting the gastrointestinal flora or digestibility of

feedingstuffs; or (7) have a coccidiostatic or histomonostatic effect; and shall be allocated to one or more of the following categories.

### *Technological additives*

*Technological additives* is defined as any substance added to feed for a technological purpose, and include the following functional groups: (1) preservatives: substances or, when applicable, microorganisms which protect feed against deterioration caused by microorganisms or their metabolites; (2) antioxidants: substances prolonging the storage life of feedingstuffs and feed materials by protecting them against deterioration caused by oxidants; (3) emulsifiers: substances that make it possible to form or maintain a homogeneous mixture of two or more immiscible phases in feedingstuffs; (4) stabilizers: substances which make it possible to maintain the physico-chemical state of feedingstuffs; (5) thickeners: substances which increase the viscosity of feedingstuffs; (6) gelling agents: substances which give a feedingstuff texture through the formation of a gel; (7) binders: substances which increase the tendency of particles of feedingstuffs to adhere; (8) substances for control of radionuclide contamination: substances that suppress absorption of radionuclides or promote their excretion; (9) anticaking agents: substances that reduce the tendency of individual particles of a feedingstuff to adhere; (10) acidity regulators: substances which adjust the pH of feedingstuffs; (11) silage additives: substances, including enzymes or microorganisms, intended to be incorporated into feed to improve the production of silage; and (12) denaturants: substances which, when used for the manufacture of processed feedingstuffs, allow the identification of the origin of specific food or feed materials.

### *Sensory additives*

*Sensory additives* is defined as any substance that improves or changes the organoleptic properties of the feed and/or the visual characteristics of the food derived from animal, and include the following functional groups: (1) colorants: (a) substances that add or restore color in feedingstuffs; (b) substances which, when fed to animals, add colors to food of animal origin; and (c) substances which favorably affect the color of ornamental fish or birds; and (2) flavoring compounds: substances, inclusion of which in feedingstuffs increases feed smell or palatability.

### *Nutritional additives*

The following functional groups are included: (1) vitamins, pro-vitamins and chemically well-defined substances having similar effect; (2) compounds of trace elements; (3) amino acids, their salts and analog; and (4) urea and its derivatives.

### **Zootechnical additives**

*Zootechnical additives* is defined as any additive used to affect favorably the performance of animals in good health or used to affect favorably the environment, and includes the following functional groups: (1) digestibility enhancers: substances which, when fed to animals, increase the digestibility of the diet, through action on target feed materials; (2) gut flora stabilizers: microorganisms or other chemically defined substances, which, when fed to animals, have a positive effect on the gut flora; (3) substances which favorably affect the environment; and (4) other zootechnical additives.

Commission Regulation (EC) No. 429/2008 (EC, 2008b) on implementing rules and guidelines concerning applications for authorization of feed additives for use in animal nutrition in accordance with Regulation (EC) No. 1831/2003 is in application; Regulation (EC) No. 429/2008 (EC, 2008b) provides specific guidelines for the authorization of feed additives. Specific guidelines have been prepared for the FEEDAP Panel of EFSA for additives that are already authorized for use in food, in minor species, as nutritional additives in pets and other non-food producing animals as well as for sensory additives other than flavoring compounds, technological additives (silage additives), technological additives other than silage additives, zootechnical additives (enzymes, microorganisms), zootechnical additives other than enzymes and microorganisms and coccidiostats and histomonostats, among others.

## COMPARATIVE SETTING TOXICOLOGICAL STANDARDS FOR FOOD SAFETY

### **Establishment of European Community maximum residue limits for drugs and feed additives**

The Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicines Agency (EMA) and Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) in order to protect human health, maximum residue limits (MRLs) for veterinary medicinal products (EEC, 1990) and for feed additives (EC, 2003) in foodstuffs of animal origin should be established in accordance with generally recognized principles of safety assessment, taking into account toxicological risks, environmental contamination and well as unintended microbiological and pharmacological effects of residues. Council Regulation 90/2377/EEC (EEC, 1990) regulates

the safety assessment for establishing the MRLs for veterinary medicinal active ingredients. From January 1992, Member States were incapable of granting marketing authorization for veterinary medicines containing new pharmacologically active substances unless an MRL has been established by the EMA-CVMP. Furthermore, the Community had set MRLs for existing pharmacologically active substances in 1997. From January 1, 1997, it was not possible to use any pharmacologically active substance in veterinary medicines intended for food animals for there is no Community MRL.

The safety or risk assessment of residues of veterinary drugs and feed additives is a science-based process involving the four following stages: (1) hazard identification; (2) hazard characterization; (3) exposure assessment ; and (4) risk characterization. Hazard is defined as the potential of a chemical agent to cause harmful effect(s) and risk as a function of the probability that an adverse effect will occur due to the presence of a hazardous compound in food and the severity of the adverse effect (exposure  $\times$  toxicity). It is not possible to completely separate the hazard identification and hazard characterization steps. For hazard identification of veterinary drugs a defined set of animal test covering the entire range of possible adverse effects is routinely required. Information on dose-dependent toxicokinetics properties (rate and extent of absorption, distribution, metabolism and excretion) in test animals, in the target animal species (and in humans when available), is always indispensable for hazard identification and characterization. If metabolites are formed in the target animal species which did not appear in the laboratory animal species, separate testing of those metabolites may be necessary. Other special studies may be required on a case-by-case basis. The standard approach to assessing the safety of chemical contaminants in foodstuffs intended for human consumption is the acceptable daily intake (ADI) defined as an estimate of the amount of a substance in food or drinking water, expressed on a body weight basis, that can be ingested daily over a lifetime without an appreciable health risk (standard human = 60 kg) and it is expressed in units of mg per kg of body weight (FAO/WHO, 2004). For the determination of the no observable adverse effect level (NOAEL), or sometimes a lowest observed adverse effect level (LOAEL), a series of doses is used. In order to establish the dose–effect relationship, the dose levels are chosen in such a way that the highest dose causes an adverse effect and the lowest dose does not produce any adverse effect. The establishment of the ADI from the determination of a NOAEL and application of an appropriate safety factor or uncertainty factor provide the hazard identification and characterization. The ADI approach was developed to take account of effects based on classical toxicology and it is applied to the results of standard toxicity

studies in laboratory animals. The NOAEL for the most sensitive toxicological parameter, normally in the most sensitive species of experimental animal, is used as the starting point (WHO, 1989). In order to arrive at such a NOAEL, a battery of standardized toxicity tests is carried out (i.e., acute toxicity, genotoxicity studies (mutagenicity, clastogenicity), subchronic oral toxicity, chronic oral toxicity/carcinogenicity and specific studies concerning reproduction and developmental toxicity among others) (EC, 2008b). The ADI was calculated by dividing this by a suitable safety or uncertainty factor, conventionally of 100, to account for the differences between test animals and humans (factor of 10) and possible differences in sensitivity between humans (another factor of 10). Depending on the available data set and the nature of substance under study higher or lower safety factors may be applied (e.g., small size of the animal test group, nature of the critical effects, quality of the data). This concept cannot be applicable in cases where certain genotoxic carcinogens do not show a dose-dependent threshold level.

ADIs are related to chemical substances which are deliberately added to a product or ingredient or which can be found on food following, for instance, treatment of crops with pesticide sprays or antifungal agents. A tolerable daily intake (TDI) is an estimate of the quantity of a chemical contaminant to which we may be exposed through environmental contamination, and which when found in food can be ingested daily over a lifetime without posing a significant risk to health.

The ADI represents the total drug residues, parent and all metabolites, that can be safely consumed daily throughout one's life. ADIs also can be developed from pharmacological or microbiological data based on no pharmacological observed effect levels. However, the impact of low levels of antibiotics on the intestinal microflora is not examined directly in these toxicological studies. For substances with microbiological activity what is actually used is the major adverse microbiological effect arising from the effects of residues of antimicrobial drugs in food of animal origin acting on the human gastrointestinal bacteria flora, particularly those acting on the colonic flora. This approach is currently used by

CVMP and JECFA (Joint FAO/WHO Expert Committee on Food Additives) but has never been used by the FEEDAP Panel as can be seen in Commission Regulation (EC) No. 429/2008 (EC, 2008b).

The use of mathematical and statistical approaches for hazard characterization appears to be valuable. One assessment approach that has been used in recent years is the use of statistical estimates based on all the available data in a dose–response series to determine the point of departure from background level response through the use of the benchmark dose (BMD) as a statistical estimate of the NOAEL. The benchmark dose is based on modeling all of the available dose–response data and generally, estimates and excess risk of 10% over background for the response of interest. The benchmark dose lower limit (BMDL) uses the lower bound of a 95% confidence limit on the benchmark dose. The BMD/BMDL approach is just one of a number of approaches that has been developed to refine the ability to characterize the toxicological hazard of a veterinary drug (Edler *et al.*, 2002).

The identification of the residue profile and its decline in the treated animal, resulting in the setting of MRLs, enables the exposure assessment and risk characterization to be determined. Additionally the theoretical maximum daily intake (TMDI) is calculated. The TMDI is an estimate of dietary intake based on multiplying the MRL by the average food consumption for each commodity (i.e., it is assumed that a person with a body weight of 60kg consumes every day over a lifetime 500g of mammalian or poultry “meat” (muscle, liver, kidney, fat) or 300g of fish plus 1500g of milk plus 100g of eggs plus 20g of honey)) and then summing the products (Table 10.6) (EMA-CVMP and WHO, 1989). It is assumed that all the previous tissues and products contain residues at a concentration equivalent to the MRL every day.

Article 14(7) of Regulation (EC) No. 470/2009 (EC, 2009a) states “where it appears necessary for the protection of human health, the classification shall include conditions and restrictions for the use or application of a pharmacologically active substance used in veterinary medicinal products which is subject to a MRL, or for which no MRL has been set” (e.g., not for use in animals

TABLE 10.6 Standard food basket

Mammals		Poultry		Fish		Bees	
Muscle	0.300 kg	Muscle	0.300 kg	Muscle and skin in	0.300 kg	Honey	0.20 kg
Fat	0.050 kg <sup>1</sup>	Fat and skin in natural proportions	0.090 kg	natural proportions			
Liver	0.100 kg	Liver	0.100 kg				
Kidney	0.050 kg	Kidney	0.010 kg				
Milk	1.500 kg	Eggs	0.100 kg				

<sup>1</sup>Fat and skin in natural proportions for pigs.

**TABLE 10.7** Conditions and restrictions for the use or application of a pharmacologically active substance used in veterinary medicinal products

Other provisions	Pharmacologically active substances
Not for use in animals from which milk is produced for human consumption (according to Article 14(7) of Regulation (EC) No. 470/2009)	Abamectin (OV); acetylsalicylic acid and acetylsalicylic acid DL-lysine (all food producing species except fin fish); aluminum salicylate (BO); apramycin (BO, OV, PO, CH, RA); bromhexine (BO); carbasalate calcium (all food-producing species except fin fish); clorsulon (BO); closantel (BO); <i>Cimicifugae racemosae rhizome</i> (all food-producing species); cyromazine (OV); decoquinate (BO, OV); derquantel (OV); dicyclanil (OV); difloxacin (BO, OV, CA); doramectin (all mammalian food-producing species); doxycycline (BO); florfenicol (BO, OV, CA); fluazuron (BO); flumethrin (OV); gamithromycin (BO); halofuginone (BO); imidocarb (OV); ivermectin (all mammalian food-producing species); levamisole (BO, OV); mebendazol (OV, CA); methylprednisolone (BO); monepantel (OV, CA); nitroxinil (BO, OV); oxolinic acid (all food-producing species); paromomycin (all food-producing species); phoxim (OV); rafoxanide (BO, OV); <i>Ruta graveolens</i> (all food-producing species); sodium acetylsalicylate and salicylate (all food-producing species except fin fish); tildiposin (BO, CA); toltrazuril sulfone (all mammalian food-producing species); tulathromycin (BO).
For milk MRL further provisions in Council Directive 94/29/EC are to be observed	Alphacypermethrin (BO, OV); cyflutrin (BO, CA); cyhalothrin (BO); cypermethrin (all ruminants); permethrin (BO).
Not for use in animals from which eggs are produced for human consumption (according to Article 14(7) of Regulation (EC) No. 470/2009)	Acetylsalicylic acid and acetylsalicylic acid DL-lysine (POU); amoxicillin (POU); ampicillin (POU); benzylpenicillin (POU); bromhexine (POU); carbasalate calcium (POU); cloxacillin (POU); danofloxacin (POU); dicloxacilin (POU); difloxacin (POU); doxycycline (POU); enrofloxacin (POU); florfenicol (POU); flumequine (POU); kanamycin (POU); levamisole (POU); oxacillin (POU); oxolinic acid (POU); paromomycin (POU); phenoxymethylpenicillin (POU); sarafloxacin (CH); sodium acetylsalicylate (POU); spectinomycin (POU); spiramycin (CH); sulfonamides (POU); thiamphenicol (POU); tilmicosin (POU); toltrazuril (POU); trimethoprim (POU); tylvalosin (POU).
Not for use in fish from which eggs are produced for human consumption (according to Article 14(7) of Regulation (EC) No. 470/2009)	Azagly-nafarelin ( <i>Salmonidae</i> ).
Only for zootechnical use and in accordance with the provisions of Directive 96/22/EC	Altrenogest (PO, <i>Equidae</i> ).
Only for intravaginal therapeutic and zootechnical use and in accordance with the provisions of Directive 96/22/EC	Progesterone (BO, OV, CA, <i>Equidae</i> (female)).
For zootechnical use only	Chlormadinone (synthetic progesterone analog) (BO).
For intravaginal use for zootechnical purposes only	Flugestone acetate (OV, CA); medroxyprogesterone acetate (OV).
For therapeutic and zootechnical purposes only	Norgestomet (bovine); 17 $\beta$ -estradiol (all mammalian food-producing species).

CA = caprine; BO = bovine; CH = chicken; OV = ovine; PO = porcine; POU = poultry; RA = rabbit

from which milk or eggs are produced for human consumption) (see Table 10.7).

Table 10.8, “prohibited substances,” includes substances considered to be unsafe on the grounds of public health (Regulation 37/2010, Table 2 of Annex) (EU, 2010).

### Maximum residue level (MRL)

All new pharmacologically active substances which are intended for use in food animals must have MRLs established prior to licensing. Termed tolerances in the U.S., is defined as the maximum concentration of a residue of a pharmacologically active substance which may be permitted in food of animal origin. In the EU those drugs for which an MRL value should be established

**TABLE 10.8** Prohibited substances in the EU

Substances	Toxicological endpoints
<i>Aristolochia</i> spp. and preparations thereof	nephrotoxicity, mutagenicity and carcinogenicity.
Chloramphenicol	aplastic anemia, carcinogenicity.
Chloroform	mutagenicity and carcinogenicity (lack of data).
Chlorpromazine	behavioral changes.
Colchicine	genotoxicity.
Dapsone	reproductive and teratogenic effects.
Dimetridazole	reproductive and teratogenic effects.
Metronidazole	mutagenicity.
Nitrofurans (including furazolidone)	carcinogenicity.
Ronidazole	mutagenicity.



are governed by Regulation (EC) No. 470/2009 (EC, 2009a), repealing Council Regulation (EEC) No. 2377/90 (EEC, 1990). According to Article 6 of this Regulation the scientific risk assessment (RA) shall consider the metabolism and depletion of pharmacologically active substances in relevant animal species, the type of residues and the amount thereof, that may be ingested by human beings over a lifetime without an appreciable health risk expressed in terms of ADI. Alternative approaches to ADI may also be used. The RA is concerned with the following: (1) the type and amount of residue considered not presenting a safety concern for human health; (2) the risk of toxicological, pharmacological or microbiological effects in human beings; and (3) residues that occur in food of plant origin or that come from the environment. If the metabolism and depletion of the substance cannot be assessed, the scientific risk assessment may take into account monitoring data or exposure data.

The standard approach to assessing the safety of residues in foodstuffs intended for human consumption is based on the determination of the ADI on which, in turn, MRLs are based. The establishment of an ADI from the determination of a NOAEL/LOAEL and application of an appropriate safety factor provide the hazard identification and characterization. The ADI approach takes account of effects based on classical toxicology. There are very potent compounds that exert pharmacological actions through receptor pathways at very low doses; the consequences are very low ADI (or reference dose, RfD) values for human dietary consumption. The  $\beta$ -adrenergic agonists are an example, so the JECFA review of the  $\beta$ -adrenergic agonist ractopamine HCl resulted in a recommendation of an ADI of 0 to 1  $\mu\text{g}/\text{kg}$  body weight/day and MRL of 10  $\mu\text{g}/\text{kg}$  of muscle with higher values in liver and kidney; by contrast this  $\beta$ -adrenergic agonist is not authorized in EU because no ADI or MRL has been set. The ADI can also be determined from microbiological data for substances with microbiological activity. To establish MRLs for a given drug requires provision of the following data: knowledge of dosage schedule (amount, dose interval and duration) and administration route; metabolic and pharmacokinetic data in laboratory animals and each of the target food producing species; distribution and residues depletion data for the major edible tissue (i.e., muscle, fat, liver and kidney) in each target species using radiolabeled drug; validated analytical methods for detection and quantitation of residues, including marker residue; and data defining the effect of residues on food processing. Under EU legislation (Article 14(2) of Regulation (EC) No. 470/2009 (EC, 2009a)) the classification of pharmacologically active substances shall also establish, in relation to each such substance, and, where appropriate, specific foodstuffs or species, one of the following: (1) an MRL; (2) a provisional MRL (pending further data); (3) the absence of the need to establish an MRL; and (4) a prohibition

on the administration of a substance. Those substances included in Annex I, II or III of and amount of residue considered not to present a safety concern for human health. Those substances included in Annex I, II or III of Council Regulation (EEC) No. 90/2377 (EEC, 1990a) are listed in the Annex of Commission Regulation (EC) No. 37/2010 (EU, 2010) (Table 1, allowed substances, where there are listed the pharmacologically active substance, marker residue, animal species, MRL value, target tissues, other provisions (according to Article 14(7) of Regulation (EC) No. 470/2009 (EC, 2009a)) and therapeutic classification, and Table 2 (prohibited substances) (where an MRL cannot be established)) (Table 10.9). This classification substitutes the four annexes of Council Regulation (EEC) No. 2377/90.

#### *Establishment of codex maximum residue limits for drugs and feed additives*

JECFA is an international expert scientific committee that is administered jointly by the Food and Agricultural Organization of the United Nations (FAO) and the World Health Organization (WHO). JECFA serves an independent scientific committee which performs risk assessments and provides advice to FAO, WHO and the member countries of both organizations. The requests for scientific advice are for the main part channeled through the Codex Alimentarius Commission (CAC) in their work to develop international food standards and guidelines under the Joint FAO/WHO Food Standards Programme. The main purposes of this program are protecting health of the consumers and ensuring fair trade practices in the food trade. The advice to CAC on residues of veterinary drugs is provided by the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF). FAO and WHO

**TABLE 10.9** Active substances are classified under four categories

Regulation (EC) 470/2009	Regulation (EEC) 2377/90
(a) A maximum residue limit.	Annex I. List of pharmacologically active substances for which maximum residue limits have been fixed.
(b) A provisional maximum residue limit.	Annex III. List of pharmacologically active substances used in veterinary medicinal products for which provisional maximum residue limits have been fixed.
(c) The absence of the need to establish a maximum residue limit.	Annex II. List of substances not subject to maximum residue limits.
(d) A prohibition on the administration of a substance.	Annex IV. List of pharmacologically active substances for which no maximum levels can be fixed.

have complementary functions in selecting experts to serve on the Committee. For residues of veterinary drugs, the WHO panel of the Joint Expert Committee is responsible for the toxicological evaluations of the substances under consideration, in order to establish ADIs (or provisional ADIs) when possible. The FAO panel develops specifications for the identity and purity of substances, assessment of residue levels of veterinary drugs in food and quality of the monitoring data; it also proposes limits (MRLs or provisional MRLs) for residues of veterinary drugs in products of animal origin, based on the WHO ADIs and on information about the distribution of the residues in tissues of the target animal. In setting the MRLs, the TMDI is estimated using the exaggerated consumption package for products of animal origin. Veterinary drug residues include parent drugs as well as their metabolites; the metabolites are taken into account if they are toxicologically relevant, i.e., present in a considerable quantity or having a toxicological or pharmacological potential. The MRL is expressed in terms of parent drug levels or in terms of levels of a marker metabolite, if the percentage of the marker metabolite formed from the parent drug is known.

#### *Changes in calculation of MRLs*

In the new JECFA approach, median residue concentration levels are used to derive an estimated daily intake rather than the MRL to better reflect estimates of chronic (lifetime) exposure (WHO, 2006). The MRL and the median concentration are derived from the same time point of the depletion data of the marker residue. The MRL itself is a point on the curve describing the upper one-sided 95% confidence limit over the 95th percentile and the median is the corresponding point on the regression line for the same time point. The JECFA concluded that the TMDI is no longer used as the most suitable estimate of chronic intake. The new procedure uses the same formula as used previously for the calculation of the TMDI, including factors such as the ratio of marker to total residue concentrations; the only exception is the median concentration that replaces the MRL as the point estimate of the residue concentration. Summarizing, the EU throughout the EMA should consider carefully the new MRL JECFA proposals because some impact is expected in the existing MRL assessments.

As indicated in the modification draft of Council Regulation (EEC) No. 2377/90 (EEC, 1990), the European Community contributes in the context of the *Codex Alimentarius* to the development of international standards on MRLs, while ensuring that the high level of human health protection adopted in the European Community is not reduced. The European Community should therefore take over, without a further risk assessment, those Codex maximum residue limits it

has supported in the relevant Codex Alimentarius Commission meeting. Consistency between international standards and Community legislation on residue limits in food will thereby be further enhanced.

With the modification of Council Regulation (EEC) No. 2377/90 (EEC, 1990) a provisional MRL may be established for a pharmacologically active substance in cases where scientific data are incomplete, provided that there are no grounds for supposing that residues of the substance concerned at the level proposed present a hazard for human health

#### *United States of America Food and Drug Administration tolerance setting*

The FDA does not regulate separately the drugs for food-producing animals from the feed additives for animal nutrition. The toxicological assessment of a veterinary drug or a feed additive establishes the basis for identifying the most appropriate *in vitro* and *in vivo* study upon which a no observable effect level (NOEL) and, subsequently, an ADI can be established. Based on the quantity and quality of the data and the scientific interpretation of those data, a safety factor is applied to the NOEL to establish the ADI. The second major component described in that guidance (FDA, 2003a) in regards to safety evaluation is exposure to humans from consumption of residues of toxicological concern in tissues (and milk and eggs, as appropriate). That process begins with metabolism and radiolabeled residue studies in the target animals. The three principal components of this endeavor include comparative metabolism in the toxicological species, total radiolabeled residue and metabolism studies in the food-producing animals, and residue depletion studies to establish the pre-slaughter withdrawal period and, when needed, the milk withdrawal and discard time. Total residue and metabolism study provides information to establish the appropriate marker residue (is the parent drug or any of its metabolites or a combination of any of these with a known relationship to the concentration of the total residue in each of the various edible tissues at the expected withdrawal time) and to determine the target tissue (represent the edible carcass from which residue depletes most slowly and is the edible tissue selected to monitor for the marker residue in the target animal). For FDA, and following the guidance above when the tolerance is based on a toxicological endpoint, the concentration of the marker residue (i.e., the compound used to monitor the depletion of total residue in a food-animal tissue) in the target tissue (i.e., the edible tissue from which the residue depletes most slowly) at the time the total radiolabeled residues in the target tissue have depleted to less than the target tissue in safe concentration, is the target tissue tolerance (word similar to maximum residue limits). When the ADI is based

on a microbiological endpoint (FDA, 2003b), a similar approach is used; however, only residues of microbiological concern are considered. In the FDA approach, when the concentration of the marker residue in the target tissue is less than the target tissue tolerances, total residues in all the edible tissues are less than their respective safe concentrations (i.e., the entire food animal carcass is safe). Safe concentrations of residues in the animal tissues are related to the ADI and food consumption factors. FDA uses the same food consumption factors as are used in the JECFA; however, they are applied differently (Ellis, 2004). FDA assumes that if a person consumes 300 g of muscle tissue, he/she will not consume an allocation of liver or kidney tissue but may consume a full allocation of milk and eggs. The food allocation is 300 g for muscle, 100 g for liver, 50 g for kidney and fat, 1500 g for milk and 100 g for eggs. Therefore, for a safe concentration in muscle for a 60 kg consumer, the ADI (in mg/kg, for example) is multiplied by 60 kg and that value is divided by 0.3 kg. For liver, the denominator will be 0.1 kg and for kidney and fat the denominator will be 0.05 kg. The daily intake of meat products is taken as being one third of the total solid diet of 1500 g (i.e., 500 g). For milk the denominator is 1.5 kg and for eggs it is 0.1 kg.

### Drug withdrawal/withholding periods

A critical factor in the medication of all food-producing animals is the mandatory withdrawal period, defined as the time during which drug must not be administered prior to the slaughter of the animal for consumption. The withdrawal period is an integral part of the regulatory authorities' approval process and is designed to ensure that no significant drug residue is present in the animal at slaughter. Drug residues in food-producing animals should comply with the MRL values for their target tissues in the animal species. The withdrawal period is intended to ensure that no harmful residues remain in edible tissues after slaughter and it is usually established as the slaughter time when residue levels in all the edible tissues are below the MRL. Adherence to the withdrawal period provides assurance that food derived from treated animals will not exceed the MRL (termed tolerances in the USA) for the drug substance. Failure to keep the pre-slaughter withdrawal period while using animal drug is the major cause of violative drug tissue residues in food-poultry production in the EU. Even if the withdrawal period involves only a few days or a few hours, the resulting residues can violate the national regulations against sale of adulterated foodstuffs which can originate distortions of competition between Member States of the EU. Withdrawal period, based on the MRL, is fixed by the regulatory authorities and will take into account the use of veterinary drugs in avian species. For example, for the determination of

the withdrawal period in avian species, six animals per slaughter time are needed. An appropriate withdrawal period is then established to ensure that the residues in edible tissues are depleted below the MRLs. A withdrawal period should be established for the substances with MRLs included in Annex (Table 1) of Regulation (EC) No. 37/2010 (EU, 2010). EMA recommend the harmonization of withdrawal periods using linear regression analysis of log-transformed tissue concentrations determined at the time when the 95% upper one-side tolerance limit was below the MRL with 95% confidence (Anadón *et al.*, 2008a, b, Anadón *et al.*, 2011).

In the EU a veterinary prescription is required for veterinary products for food-producing animals. If it is necessary for a VMP to be prescribed for a species for which it is not licensed or at a dosage higher than the licensed dosage, the veterinarian will specify an appropriate withdrawal period. In exceptional cases, where no medicine is authorized, there is a possibility for the veterinarian to use, for example, products that are authorized in other EU countries or for other animal species. This exception exists to avoid unacceptable suffering of animals. In these cases the veterinarian has to follow specific steps, the so-called "cascade", and has to make sure that there is no risk for the animal(s) concerned and for consumers of food products of animal origin. The exceptional off-label use of authorized medicines is allowed under specific conditions described in Article 11 of Directive 2004/28/EC (EC, 2004b), which are often referred to as the "cascade." EU Member States are obliged to take the necessary measures to ensure that, if there is no authorized VMP in a member state for a specific condition affecting a food-producing species, by way of exception, the responsible veterinarian may, under his/her direct personal responsibility and in particular to avoid causing unacceptable suffering, treat the animals concerned following the algorithm indicated in Table 10.10.

The veterinarian may administer the medicinal product personally or allow another person to do so under the veterinarian's responsibility. For food-producing animals, these provisions apply to animals on a particular holding only; the pharmacologically active substances in the medicinal products used must be listed in the Annex of Regulation No. 37/2010 (Table 1, allowed substances) (EU, 2010), and the veterinarian must specify an appropriate period, which shall be at least 7 days for eggs, 7 days for milk, 28 days for meat from poultry and mammals, including fat and offal, and 500 degree-days for fish meat.

## INDUSTRIAL CHEMICALS

The area of regulations covering industrial chemicals is one of increasing harmonization within the EU. This is

TABLE 10.10 Exceptional off-label use of authorized medicines (“cascade” system)

No authorized veterinary medicinal product (VMD) in a member state for a specific condition	
(a) A VMP authorized in the member state concerned for use for another animal species, or for another condition in the same species; or	
<i>Medicinal product</i>	<i>Medicinal product or imported VMP</i>
(b) A medicinal product for human use authorized in the member state concerned	(b) If there is no such product authorized, either: (i) a medicinal product for human use authorized in the member state concerned, or (ii) a VMP authorized in another member state for use in the same species or in another food-producing species for the condition in question or for another condition may be used;
(c) If, however, there is no such product, a VMP prepared extemporaneously by a person authorized to do so following a veterinary prescription may be used.	

particularly true with regard to the need for providing adequate toxicity data on new chemicals, to allow any potential hazard to humans to be identified, and in the area of labeling of dangerous substances and preparations (i.e., mixtures of substances). A key Directive is Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labeling of dangerous substances, commonly referred to as the Dangerous Substances Directive (EEC, 1967) and its subsequent amendments as well as Council Regulation (EEC) No. 793/93 of March 23, 1993 on the evaluation and control of the risks of existing substances (EEC, 1993). On the other hand, REACH is the Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (EC, 2006). It entered into force on June 1, 2007. It streamlines and improves the former legislative framework on chemicals of the EU. The main aims of REACH are to ensure a high level of protection of human health and the environment from the risks that can be posed by chemicals, the promotion of alternative test methods, the free circulation of substances on the internal market and enhancing competitiveness and innovation. REACH makes industry responsible for assessing and managing the risks posed by chemicals and providing appropriate safety information to their users. In parallel, the EU can take additional measures on highly dangerous substances, where there is a need for complementing action at EU level. All manufacturers and importers of chemicals must identify and manage risks linked to the substances they manufacture and market. For substances manufactured or imported in quantities of 1 ton or more per year per company, manufacturers and importers need to demonstrate that they have appropriately done so by means of a registration dossier, which must be submitted to the European Chemicals Agency (ECHA). The ECHA may then check that the registration dossier complies with the Regulation and must evaluate testing proposals to ensure that the assessment of the chemical substances will not result in unnecessary testing, especially on animals, but also that adequate information is provided. Where appropriate, authorities may also select

substances for a broader substance evaluation to further investigate substances of concern. Six substances of very high concern will be banned within the next 3 to 5 years unless an authorization has been granted to individual companies for their use. These substances are carcinogenic, toxic for reproduction or persist in the environment and accumulate in living organisms. The following substances are of concern: musk xylene, MDA, HBCDD, DEHP, BBP and DBP.

REACH also foresees an authorization system aiming to ensure that substances of very high concern are properly controlled and progressively replaced by suitable alternative substances or technologies where these are economically and technically viable. Where this is not possible, the use of substances may only be authorized where there is an overall benefit for society of using the substance. In addition, EU authorities may impose restrictions on the manufacture, use or placing on the market of substances causing an unacceptable risk to human health or the environment. The EU Member States authorities are responsible for enforcing REACH through inspections as well as penalties in case of non-compliance. From June 1, 2009, Annex XVII of the REACH Regulation replaced Directive 76/769/EEC (EEC, 1976) on the approximation of the laws, regulations and administrative provisions of the Member States, relating to restrictions on the marketing and use of certain dangerous substances and preparations

## CONCLUSIONS

Complicated and extensive systems for the marketing authorization of veterinary medicinal products and other chemical compounds exist in the EU. These systems attempt to ensure the quality, efficacy (including an objective of overall assessment of the risk/benefit balance of the final product) and safety of veterinary drugs and feed additives. Safety to consumers is of enormous importance and this demands a meticulous assessment of pharmacological, toxicological and residues data.



The marketing authorization procedure for veterinary medicinal products in the EU is of much interest in terms of risk assessment of residues. The main human health risks from drug residues in foods are acute toxic effects and long-term chronic effects. Carry-over or cross-contamination of the feed by coccidiostats can result in the exposure of non-target animal species and induce adverse effects. The pharmacologically active substances contained in biocidal products or in feed additives used in animal husbandry should be submitted to the establishment of MRL as indicated in the current EU regulations. The comparative setting of toxicological standards for food safety are very useful to raise proposal on safety evaluation strategies. Failure to keep the pre-slaughter withdrawal period while using animal drugs is the major cause of violative drug tissue residues in food-poultry production in the EU.

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# Safety evaluation of new molecular entities for pharmaceutical development

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## INTRODUCTION

It is well known that the development of a new molecular entity (NME) into a safe and efficacious pharmaceutical product typically may take about one billion dollars in revenues and up to 15 years to launch the product in the market. However, the exceptions are made for entities developed for rare conditions, occurring in small populations (fewer than 200,000 patients) that are classified as orphan drugs and reviewed by the United States Food and Drug Administration (US FDA) under fast-track application. In addition, the drug candidates for indications in life-threatening or serious diseases (e.g., advanced cancer, resistant human immunodeficiency virus (HIV) infection and congenital enzyme deficiency diseases) are also reviewed under fast-track application and the approval process may not require a traditional pharm/tox package during safety reviews.

A number of good books on preclinical development have been published by renowned experts. In addition, there are a large number of regulatory guidelines available to recommend specific studies for pharmacology and toxicology section of the regulatory applications for global submissions and marketing authorization. In the United States, the submission are made to the U.S. Food and Drug Administration (USFDA) and primarily reviewed by either the Center for Drug Evaluation and Research (CDER) or the Center for Biologics Evaluation and Research (CBER). The preclinical safety information is submitted initially in the pharmacology/toxicology section of an investigational new drug (IND) application and later updated with additional chronic and specific toxicology studies in the pharmacology/toxicology

section of the new drug application (NDA), biologics license application (BLA), 510K submission for medical devices or through an abbreviated new drug application (505(b)(2) submission) for a molecular entity that has been previously approved through regulatory process and for which some supporting safety data have been reviewed. The safety data are included and electronically submitted as part of a common technical document (CTD). A CTD contains five modules, in which module 2 consists of non-clinical overview and nonclinical tabulated summaries and module 4 consists of full GLP toxicity reports.

Since 1990, the three regions (U.S., Europe and Japan) have harmonized the requirements for pharmaceutical development; therefore, most of the guidelines follow International Conference on Harmonization (ICH) rules and are considered as ICH guidelines. These ICH guidelines comply with both GLP regulations (21 CFR Part 58, April 2011) as well as with guidelines per Office of Economic Cooperation and Development (OECD, 2004–2006).

The objective of this chapter is to introduce the process of pharmaceutical development and further describe the safety evaluation of NMEs as potential therapeutic agents, in a precise and comprehensible manner. The contents of this chapter may include some information from publicly available regulatory guidelines; however, the complete references have been cited in the reference section and can be consulted for any further detailed information. The chapter does not describe the study designs of GLP toxicity studies since the information is extensive and, therefore, cannot be accommodated in this chapter. The chapter also does not describe all the safety requirements for medical devices.

The development of a pharmaceutical agent is a step-wise process involving an evaluation of both animal and

human efficacy and safety information. Drug development can be broadly classified into (1) preclinical and (2) clinical developments. The first step of preclinical development is known as target discovery, which involves lead identification and where the molecular entity must target a therapeutic mechanism. Once these NMEs are identified, they are validated and optimized for their potency. The discovery process usually involves the use of *in vitro* models. Among hundreds and thousands of molecules, only a handful of potent molecules are able to get through non-clinical and clinical development to establish their safety and efficacy as potential therapeutic agents. The second step of preclinical testing is known as nonclinical testing, in which the lead candidate(s) undergoes thorough safety evaluation in animal models per GLP regulations (21 CFR Part 58, April 2011). Based on the nonclinical data, they are further tested in the clinical trials for their clinical safety, efficacy and marketing authorization.

Typical safety studies are expected to be performed in compliance with Good Laboratory Practice (GLP) regulations for U.S. submissions and per OECD guidelines for European and Japanese submissions. In cases where non-compliance might have occurred, those areas need to be identified and their effect on the outcome of overall safety assessment determined. The lack of full GLP does not necessarily mean that the data from these studies cannot be used to support clinical trials and market authorizations.

## NONCLINICAL SAFETY EVALUATION

A therapeutic agent can be classified as either (1) small molecule, that is chemical in nature and has a molecular weight of less than 10,000Da, (2) biologic agent (larger than 10,000Da, e.g., peptides, protein or DNA derived from recombinant technology or vaccine, oligonucleotides, monoclonal antibodies, etc.) or (3) a medical device either by itself or as a drug-delivery combination.

For small molecules, a standard genotoxicity testing battery is required to determine any potential for possible mutagenicity and carcinogenicity, early in the development. The test battery typically includes two *in vitro* assays (Bacterial Reverse Mutation Assay or Ames test and chromosomal aberration assay or mouse *tk*-lymphoma test) and one *in vivo* mouse micronucleus induction assay. More details of these studies are provided later in the genotoxicity section. The earlier genotoxicity results can be provided for regulatory review in either a pre-IND or IND submission to discuss further plans and make a go/no-go decision. These studies are conducted per GLP regulations as described in genotoxicity guidance ([Guidance for Industry S2B, 1997](#)).

In addition, *in vitro* safety pharmacology studies are also recommended very early in the development ([Guidance for Industry, 57A, 2001 and 57B, 2005](#)). For this purpose, the functional testing of a potassium ion channel, the human ether-a-go-go (HERG) test, is conducted to rule out the possibility of molecular entity, causing the blockage of HERG ion channel which may lead to fatal arrhythmia (torsades de pointes). More details of these tests are provided later in the safety pharmacology section. Exceptions are made for biotechnology-derived products that are not recommended for genotoxicity and *in vitro* safety pharmacology testing.

A nonclinical studies' package includes pharmacokinetic/toxicokinetic studies, acute (single dose), subchronic and chronic (repeat-dose) studies, safety pharmacology, genotoxicity, developmental and reproductive toxicity (DART) studies (based upon requirement), and 2-year carcinogenicity studies. Pharmacology studies can be divided into three categories: primary pharmacodynamics, secondary pharmacodynamics and safety pharmacology.

The objectives of the preclinical safety studies are to define pharmacological and toxicological effects, not only prior to initiation of human studies, but throughout clinical development. The safety evaluation of either a small molecule or a biologic agent generally includes the characterization of toxic effects, with respect to target organs, dose dependence, relationship to exposure and, when appropriate, potential reversibility ([Guidance for Industry M3\(R2\), 2010](#)). Additional specific studies may include phototoxicity studies, immunotoxicity studies, juvenile animal toxicity studies and abuse potential studies, depending upon the intended use.

When conducting safety evaluation, prior safety data from the molecular entities from the same class of compounds with similar structure are always considered to support the safety of the molecular entity of interest. For biotechnology-derived products, appropriate nonclinical safety studies should be determined ([Guidance for Industry ICH S6 \(1997\) and S8 \(2006\)](#)).

Safety evaluation under GLP and OECD guidances requires that the test material should be manufactured per GMP regulations ([Guidance for Industry, CGMP Regulations, September 2006](#)) and must be characterized for its identity, strength, purity, stability over 2 years or longer at room temperature and presence of any impurities. In order to be compliant, it is mandatory to conduct dose concentration analysis (DCA) and stability of test material under conditions of use in any GLP toxicity study. Non-clinical safety testing, in general, should be conducted with consideration to (1) relevant species, (2) age, (3) physiological state, (4) dose, route of administration and clinical treatment regimen, and (5) dose-concentration analysis and stability of the test material under the conditions of use. The relevance of species is especially important for biologic agents that should demonstrate immunogenicity.



## Pharmacokinetic and toxicokinetic studies

Pharmacokinetic/toxicokinetic (PK/TK) analysis is usually conducted in all GLP toxicology studies, including single dose, repeat dose, *in vivo* genotoxicity, reproductive toxicity and carcinogenicity studies ([Guidance for Industry ICH S3A, 1995](#)). The objective of these studies is to determine systemic exposure of a parent compound and/or its metabolites, in relation to dose and time. The main parameters include area under the curve (AUC), maximum concentration ( $C_{\max}$ ) and half-life ( $t_{1/2}$ ) in which time half of the drug is removed from the blood. Blood sampling is usually conducted at short intervals in the first 2 hours and then at larger intervals up to 72 hours. Calculations of these parameters are generally done using Win-Nonlin analysis.

Information on pharmacokinetics (PK) and determination of metabolites through absorption, distribution, metabolism and excretion (ADME) testing (in animal species and *in vitro* systems) are obtained prior to Phase III human clinical trials. Such data are helpful in determining dosing frequency and duration in humans. Nonclinical characterization of a human metabolite(s) is warranted when that metabolite(s) is observed at the exposures greater than 10% of total drug exposure and at significantly greater levels in humans than the maximum exposure seen in the toxicity studies. Such studies should be conducted to support Phase III clinical trials.

For monoclonal antibodies, the immunological properties of the antibody, including its antigenic specificity, complement binding and any unintentional reactivity and/or cytotoxicity towards human tissues distinct from the intended target, should be described. Such cross-reactivity studies should be carried out by appropriate immunohistochemical procedures using a range of human tissues.

## Biological activity/pharmacodynamics (PD)

In general, primary PD studies (*in vivo* and *in vitro*) are intended to investigate the mode of action and/or effects of a substance in relation to its desired therapeutic target. Such studies are generally conducted during the discovery phase of pharmaceutical development and as such are not generally conducted in accordance with Good Laboratory Practices (GLP, 21 CFR Part 58).

Biological activity may be evaluated using *in vitro* assays to determine which effects of the product may be related to the clinical activity.

## Safety pharmacology studies

Safety pharmacology and pharmacodynamics (PD) studies are defined in the [Guidance for Industry ICH S7A](#)

(2001) and [S7B \(2005\)](#). The core battery of safety pharmacology studies includes the assessment of effects on the cardiovascular system, central nervous system and respiratory, renal and gastrointestinal systems, and are recommended to be conducted prior to human exposure. However, when warranted, follow-up safety pharmacology studies can also be conducted during later clinical development. In addition to animal models, *in vitro* and *ex vivo* systems are routinely used that include isolated organs and tissues, cell cultures, cellular fragments, sub-cellular organelles, receptors, ion channels, transporters and enzymes. However, for biotechnology-derived products, *in vivo* studies provide more meaningful data to support their use in humans. The purpose of safety pharmacology studies is to investigate the potential undesirable effects of a substance on physiological functions in the therapeutic range and above. Prior to conducting safety pharmacology studies, some related parameters can be incorporated in the design of general toxicology, PK/TK and clinical studies.

Whether using an *in vitro*, *ex vivo* or *in vivo* model, it is important to define the dose relationship of the adverse effects. In animal models, the dosing route should be as close to the intended clinical route as possible. These studies are performed by single-dose administration and the effects of parent compound and/or its metabolite(s) are evaluated and the results may vary based upon gender.

A list of safety pharmacology tests is described below. Additionally, if the concerns arise for either core battery testing, clinical trials or pharmacovigilance, the follow-up pharmacology studies (listed within parentheses) can be conducted:

1. *Central nervous system.* Motor activity, behavioral changes, coordination, sensory/motor reflex responses and body temperature are evaluated using functional observation battery (FOB; [Mattsson et al., 1996](#)), modified Irwin's test ([Irwin, 1968](#)) or other appropriate tests ([Haggerty, 1991](#)). (Follow-up studies: behavioral pharmacology, learning and memory, ligand-specific binding, neurochemistry, visual auditory and/or electrophysiological examination.)
2. *Cardiovascular system.* Blood pressure, heart rate and electrocardiogram (ECG) are evaluated. (Follow-up studies: cardiac output, ventricular contractility, vascular resistance, the effects of endogenous and/or exogenous substances.)
3. *Respiratory system.* Respiratory rate and other respiratory functions (e.g., tidal volume ([Murphy, 1994](#)) or hemoglobin oxygen saturation) are evaluated. (Follow-up studies: airway resistance, compliance, pulmonary arterial pressure, blood gases and blood pH.)
4. *Renal/urinary system.* Urinary volume, specific gravity, osmolarity, pH, fluid/electrolyte balance, proteins,

cytology and blood chemistry determinations, such as urea nitrogen, creatinine and plasma proteins.

5. *Autonomic nervous system.* Binding of receptors relevant to the autonomic nervous system, functional responses to agonists/antagonists both *in vitro* and *in vivo*, direct stimulation of autonomic nerves and measurement of cardiovascular responses, baroreflex testing and heart rate variability may be used.
6. *Gastrointestinal system.* Gastric secretion, gastrointestinal injury potential, bile secretion, transit time *in vivo*, ileal contraction *in vitro*, gastric pH measurement and pooling.
7. *Other organ systems.* Dependency potential, skeletal muscle, immune and endocrine functions.

Safety pharmacology studies are usually not required for dermal, ocular products or drugs for end-stage cancer patients.

The safety assessment on ventricular repolarization and any proarrhythmic risk is one of the most important aspects of safety evaluation. The QT interval (time from the beginning of the QRS complex to the end of the T-wave) of the electrocardiogram (ECG) is a measure of the duration of ventricular depolarization and repolarization. QT prolongation can either be congenital or induced by pharmaceutical agents. When ventricular depolarization is delayed and the QT interval is prolonged, there is an increased risk of ventricular tachyarrhythmia, including torsades de pointes. The most common mechanism of QT interval prolongation is inhibition of the delayed rectifier potassium channel responsible for  $I_{Kr}$  encoded by human ether-a-go-go (HERG) ion channel protein.

*In vitro* studies for QT prolongation can be conducted using isolated animal or human cardiac myocytes, cultured cardiac cell lines or heterologous expression system for cloned human ion channels and can address proarrhythmic effects. However, animal models allow much more precise testing of ventricular repolarization or associated arrhythmias. Laboratory animal species used for the *in vivo* electrophysiological studies include dog, monkey, swine, rabbit and sometimes guinea pig and ferret. The use of rodents is not recommended, due to the difference in ionic mechanisms of repolarization in adult rats and mice. The dose range should include a dose exceeding the anticipated human exposure but can be adjusted, taking into account any intolerance such as tremors, emesis or hyperactivity.

## General toxicity studies

For safety testing of NMEs, the recommended mammalian species include a rodent and a non-rodent model (e.g., rats and dogs). However, safety evaluation for

biologics usually includes the use of relevant species, in which the test material is pharmacologically active (shows immunogenicity), due to the expression of the receptor or an epitope (in the case of monoclonal antibodies) and demonstrate a similar tissue cross-reactivity profile as for humans. Although two relevant mammalian species are required for safety evaluation programs, in some cases only one relevant species may be sufficient. In the absence of relevant species, the use of transgenic animals expressing the human receptor or the use of homologous protein is considered.

The number of animals used per dose has a direct bearing on the ability to detect toxicity. It is advisable to use at least 10 mice per group or five rats per group, in order to be able to obtain meaningful toxicological data in acute single-dose toxicity studies, especially when the test material is available in small quantities. The suggested number may take into account any possible deaths at high doses, but still reveal statistically meaningful data. Also, a satellite group of animals for any pharmacokinetic/ADME studies would facilitate sufficient sampling for pharmacokinetics as well as clinical chemistry. Both males and females should be used unless the clinical indication is gender specific. The route and frequency of administration should be as close as possible to proposed clinical use. Consideration must be given to the pharmacokinetics and bioavailability of the product in the species being used and to the volume which can safely and humanely be administered to the test animals. In some cases, an alternative to the clinical route may be acceptable provided the modification was done, due to limited bioavailability or limitations due to the route or size/physiology of the animal species.

## Acute toxicity studies

Single-dose studies may generate useful data to describe the relationship of dose to systemic and/or local toxicity. These studies are conducted non-GLP and the highest doses of 2000 mg/kg/day for rodents and 1000 mg/kg/day for non-rodents are usually considered appropriate. The dose selection may also be based upon a 50-fold margin of exposure (usually based on the group mean area under the curve (AUC)) of parent drug or the pharmacologically active molecule of a pro-drug.

Historically, acute toxicity information has been obtained from single-dose toxicity studies in two mammalian species (one rodent and another non-rodent) using both clinical and parenteral routes of administration to define a maximum tolerated dose (MTD) in test species. Acute toxicity results could be useful to support Phase III clinical trials and to predict the consequences of possible human overdose (e.g., depression, pain and dementia). Further details are provided later in the abuse potential section.

### Subchronic and chronic toxicity studies

Dose levels for repeat-dose subchronic and chronic studies are selected based upon MTD values from acute toxicity studies, in order to provide information on a dose–response relationship, including a toxic dose, and to determine a no observed adverse effects level (NOAEL). Generally in repeat-dose toxicity studies, the clinically relevant effects can be adequately characterized, using a range of doses up to MTD or the maximum feasible dose (MFD), achieved by large exposure multiples or saturation. MTD/MFD levels are especially important for repeat-dose developmental and reproductive toxicity (DART) and carcinogenicity study designs to select less toxic doses.

NOAEL is used to calculate first safe human equivalent dose (HED) for Phase I clinical trials in healthy volunteers and details are provided in the estimation of first dose in human section.

The route and dosing regimen (e.g., daily versus intermittent dosing) is similar to the intended clinical use and often includes PK/TK analyses. The inclusion of a recovery period (especially in biopharmaceuticals) in study designs is important to determine the reversal or potential worsening effects, and/or any delayed toxic effects.

Usually 28-day repeat-dose toxicity studies in two species (one rodent and another non-rodent) may support any clinical trial up to 2 weeks. For therapeutic agents in chronic indication, 6 months' rodent and 9 months' non-rodent studies generally support dosing for longer than 6 months in clinical trials. This duration of animal dosing has generally been 1–3 months for most biotechnology-derived pharmaceuticals. For biopharmaceuticals intended for short-term use (e.g., less than or up to 7 days) and for acute life-threatening diseases, repeat-dose studies up to 2 weeks' duration have been considered adequate to support clinical studies as well as marketing authorization. The recommended duration of repeat dose toxicity studies to support the conduct of clinical trials is described in Table 11.1. The durations of repeat-dose toxicity studies to support marketing is described in Table 11.2.

### Local tolerance studies

It is preferable to evaluate local tolerance, by the intended therapeutic route, as part of general toxicity studies. In cases where the anticipated systemic exposure (area under the curve (AUC) and maximum plasma concentration ( $C_{max}$ )) is covered by the existing toxicology package, the endpoints in the local tolerance study can be confined to clinical signs and macroscopic and microscopic examination of the application site.

TABLE 11.1 Recommended duration of repeat-dose toxicity studies to support the conduct of clinical trials

Maximum duration of clinical trials	Recommended minimum duration (rodents)	Recommended minimum duration (non-rodents)
Up to 2 weeks	2 weeks <sup>a</sup>	2 weeks <sup>a</sup>
Between 2 weeks and 6 months	Same as clinical trial <sup>b</sup>	Same as clinical trial <sup>b</sup>
>6 months	6 months <sup>b,c</sup>	9 months <sup>b,c,d</sup>

<sup>a</sup>In the U.S., alternate extended single-dose toxicity studies can support single-dose human clinical trials.

<sup>b</sup>Clinical trials of longer than 3 months can be initiated if 3-month rodent and non-rodent studies are available.

<sup>c</sup>In case pediatric population is the intended population, long-term testing in juvenile animals can be conducted.

<sup>d</sup>In the EU, 6-month non-rodent studies are acceptable but no need to conduct additional 6-month study for longer duration. In the U.S. and Japan, 6-month non-rodent studies are acceptable if immunogenicity occurs, for treatment of migraine, erectile dysfunction and herpes simplex, or for drugs for life-threatening conditions such as cancer.

TABLE 11.2 Recommended duration of repeated-dose toxicity studies to support marketing

Duration of indicated treatment	Rodent	Non-rodent
Up to 2 weeks	1 month	1 month
>2 weeks to 1 month	3 months	3 months
>1 month to 3 months	6 months	6 months
>3 months	6 months	9 months <sup>c,d</sup>

N.B. Refer to notes c and d in Table 11.1.

For parenteral products, the evaluation for local tolerance at injection sites is conducted before large Phase III clinical trials. Local tolerance studies are generally not recommended in the United States. Japan and the European Union (EU) recommend single-dose paravenous administration for the intravenous route.

### Genotoxicity studies

Genotoxicity tests conducted *in vitro* and *in vivo* are designed to test any genetic damage caused directly or indirectly. Any damage to DNA in the form of gene mutations, large -scale chromosomal damage, recombinant and numerical chromosome changes is considered essential for heritable effects and in the multistep process of malignancy. Compounds that cause such changes have a potential to cause cancer in humans.

For registration of pharmaceuticals, a standard three-test genotoxicity battery is used (Guidance for Industry ICH S2B, 1997) that consists of (1) bacterial reverse mutation assay or Ames test conduct using *Salmonella* strains, (2) *in vitro* chromosomal aberrations (structural and

numerical) test conducted either using bone marrow or peripheral blood from animals or humans (alternatively, mouse *tk*-lymphoma assay may also be conducted to detect gene mutation and clastogenic effects) and (3) *in vivo* micronucleus induction test in mice that may also include additional ADME analysis to determine if a metabolite may cause such induction of micronuclei.

The negative results in this test battery usually provide a sufficient level of the absence of genotoxic potential. However, compounds giving positive results in this standard battery may have to be evaluated more thoroughly. Even if a compound is tested negative in this battery but shows any evidence of tumor response, it needs more extensive testing.

Completion of genotoxicity battery is required before the initiation of Phase II trials. The type of genotoxicity studies routinely conducted for small molecules are not applicable to biotechnology-derived pharmaceuticals.

## Carcinogenicity studies

Conditions relevant for carcinogenicity testing are discussed in the [Guidance for Industry ICH S1C\(R2\) \(2008\)](#) document and conducted to support marketing application. Typically, when bacterial reverse mutation assay and an *in vivo* genotoxicity assay are tested positive, the carcinogenicity studies may be required as a Phase IV commitment and be conducted for post-marketing approval. However, a lengthy clinical study duration for chronic indication alone is not considered to be a significant reason for these studies. For pharmaceuticals developed to treat certain serious diseases for adults or pediatric patients, carcinogenicity testing, if recommended, can also be concluded for post-marketing approval. Recently, the medical devices intended for long-term use have also been requested to be tested in carcinogenicity studies.

For biologics, standard carcinogenicity bioassays are generally considered inappropriate. However, the products (e.g., growth factors, immunosuppressive agents, etc.) that may have the potential to support or induce proliferation of transformed cells and clonal expansion, possibly leading to neoplasia, should be evaluated in a rodent model. Standard 2-year carcinogenicity testing is required using mice and rats including both males and females. Alternatively, suitable transgenic rodent models can also be used in a much shorter (~9 month) study.

Irrespective of the nature of the molecular entity, careful consideration should be given to the rationale and selection of doses ([Guidance for Industry Carcinogenicity Study Protocol Submissions, 2002](#)). Prior to the design and conduct of a standard 2-species/2-year carcinogenicity testing, it is recommended to

follow the ICH guidances ([ICH S1C\(R\), 2008](#)) and for a proposed testing plan to be reviewed by the Carcinogenic Assessment Committee (CAC). For this purpose, a 90-day repeat-dose toxicity study using the same dosing route, diet and rodent strain as intended for carcinogenicity study is required. Additionally, supporting PK/TK data including the exposure (AUC) at the maximum recommended human dose (MRHD) from clinical trials, plasma protein binding and genotoxicity data are helpful.

## Developmental and reproductive toxicity (DART) studies

Guidelines for reproductive toxicity were developed by the Expert Working Group (Safety) of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use ([Guidance for Industry ICH S5A, 1994](#)). For reproductive testing, the animals are treated during defined stages of reproduction that reflect human exposure. In order to allow detection of exposure, observations should be continued through one complete life cycle, i.e., from conception in one generation through conception in the following generation. Testing can be subdivided into the following six stages:

- 1 Premating to conception (adult male and female reproductive functions, development and maturation of gametes, mating, behavior and fertilization).
- 2 Conception to implantation (adult female reproductive functions, preimplantation development, implantation).
- 3 Implantation to closure of the hard palate (adult female reproductive functions, embryonic development, major organ formation).
- 4 Closure of the hard palate to the end of pregnancy (adult female reproductive functions, fetal development and growth, organ development and growth).
- 5 Birth to weaning (adult female reproductive functions, neonate adaptation to extrauterine life, preweaning development and growth).
- 6 Weaning to sexual maturity (post-weaning development and growth adaptation to independent life, attainment of full sexual function).

The animals should be of comparable age, weight and parity at the start being young, mature adults at the time of mating with females being virgin. Usually, rats are predominantly used because of their practicality and the large amount of background knowledge. However, in embryotoxicity studies, a second mammalian species traditionally has been required and rabbit is the preferred choice as a "non-rodent" due to extensive background



knowledge, availability and practicality. Alternatively, non-human primates may also be used for the purpose.

Selection of dosages in design of the reproductive toxicity study should be based on pharmacology, acute and, if available, chronic toxicity and PK/TK studies. A repeat-dose toxicity study of about 2 to 4 weeks' duration provides a close approximation to the duration of treatment in segmental designs (Segments I (embryofetal), II (prenatal) and III (postnatal)). An evaluation of male reproductivity is advised in the repeated-dose toxicity studies. It is important to characterize and minimize the risk of unintentional exposure of the embryo or fetus when including WOCBP (women of child-bearing potential) in clinical trials. DART studies are usually required to be completed before the Phase III clinical trials begin. In all the three ICH regions, the pre- and postnatal development studies should be submitted for marketing approval.

In addition to therapies indicated for women of child bearing potential (WOCBP), DART studies are also recommended for the development of investigational and therapeutic vaccines for infectious diseases ([Guidance for Industry FDA, vaccine guidance, 2006](#)). Since, the indication of these vaccines is for immunization in adolescent and adults, there is increasing concern about the unintentional exposure of an embryo/fetus. Therefore, the recommendations pertaining to such studies are specially indicated in WOCBP and pregnant women. Since the pregnant women are usually excluded from the clinical trials, data from developmental toxicity studies in animal models may frequently present the only information regarding developmental risk to be included in the product labeling.

Vaccines are a heterogeneous class of preventive or therapeutic products that may cause an immune response(s) and prevent and/or lessen the severity of one or more infectious diseases. A vaccine may be a live attenuated preparation of bacteria, viruses or parasites, inactivated (killed) whole organisms, living irradiated cells, crude fractions or purified immunogens, including those derived from recombinant deoxyribonucleic acid (DNA) in a host cell, conjugates formed by covalent linkage of components, synthetic antigens, polynucleotides (such as plasmid DNA vaccines), living vectored cells expressing specific heterologous immunogens or cells pulsed with immunogens. Antigens may be presented plain or derived in combination with other antigens, adjuvants, additives and other excipients.

Clinical experience from pregnant women may be helpful in the evaluation of the potential for any adverse outcome on the viability and development of the offspring, help design and monitoring of appropriate non-clinical studies and for product labeling. In addition to an immune response in pregnant females, it is necessary to verify exposure of the fetus to the maternal

antibodies. Males may be included in Phase I, II and III clinical trials in the absence of non-clinical male fertility studies.

## Pediatric drugs studies

For safety evaluation of therapeutics intended for the treatment of pediatric patients, non-clinical toxicity studies are conducted using juvenile animals that can provide meaningful prediction of toxicity in pediatric patients. Such testing is limited to safety effects that cannot be adequately, ethically and safely assessed in pediatric clinical trials. The effects that pose most concern are the irreversible serious adverse effects ([Guidance for Industry, Pediatric drug products, 2006](#); [Guidance for Industry FDA, pediatric, 2006](#)). A survey conducted by the American Academy of Pediatrics showed that the majority of the drugs listed in the Physician's Desk Reference lack information on safety and/or efficacy for pediatric use (Committee on Drugs, Pediatrics, 1995). However, recent pediatric legislation, including the [Best Pharmaceuticals for Children Act \(BPCA, 2007\)](#) and the [Pediatric Research Equity Act \(PREA, 2003\)](#), have been helpful in obtaining the critical pediatric safety and efficacy information in drug product labels. Safety data from adult animals and humans are helpful in supporting juvenile animal and pediatric studies. Since developmental processes in pediatric patients may differentially affect drug pharmacokinetics and pharmacodynamics compared to adult therapeutic use, juvenile animal studies may assist in identifying postnatal developmental toxicities that are not adequately assessed in reproductive toxicity assessments.

The delicate and immature organ systems (brain, kidneys, lungs, immune system, reproductive system and gastrointestinal system) of pediatric patients, in terms of developmental changes in metabolism (including the maturation rate of Phase I and II detoxification enzyme activities), body composition (i.e., water and lipid partitions), receptor expression and function, growth rate and organ functional capacity, make them more susceptible to modifications or disruption by drugs than the adults. Some of the examples of such drugs include acetaminophen, valproic acid, chloramphenicol, inhaled corticosteroids, aspirin and lamotrigine.

Juvenile animal testing may be useful in assessing potential developmental age-specific toxicities. The toxicological assessments are done on active and inactive ingredients in addition to any excipients. Any concern for postnatal development can be addressed either in juvenile animal studies or by modified study design (e.g., modification of segment III reproductive toxicity studies to include animals of similar developmental status as the pediatric population of concern).

The pharmacokinetic analysis of clinical studies in pediatric subjects does not involve long-term exposure because they are generally less than 6 months long. However, if the drug is indicated for chronic use, some assessment of the long-term development effects in animals should be made before marketing and the initiation of long-term clinical studies. When there have been reports of adverse effects with off-label use in pediatric patients and there are inadequate data to evaluate the relationship between the drug and the adverse effects, completed juvenile animal studies are needed before initiation of pediatric clinical studies. For serious life-threatening pediatric conditions, safety studies are considered on a case-by-case basis.

Consideration should be given to the age of the intended population and the corresponding stage of postnatal development. Most drugs that are intended for pediatric use (12 years and older) have established efficacy and safety profiles in adult humans. The embryofetal development is especially sensitive to perturbation during organogenesis, in which tissues that undergo significant postnatal development are studied in juvenile animals for those specific effects, even when the primary postnatal development is studied in juvenile animals for those specific effects.

Among preferred testing species, rats and dogs have been the rodent and non-rodent species of choice. In some circumstances, minipigs, pigs and monkeys can also be used. The choice of species depends on the pharmacology, pharmacokinetics and toxicology of the therapeutic agent, comparative developmental status of the major organs of concern between juvenile animals and pediatric patients and sensitivity of the selected species to a particular toxicity. Typically, one animal species may be sufficient to evaluate toxicity endpoints for therapeutics that are well characterized in both adult humans and animals using modified perinatal and postnatal developmental studies.

Studies include the measurement of overall growth (e.g., body weight, growth velocity per unit time, tibial length), clinical observations, measurement of organ weights, gross and microscopic examinations, clinical pathology, assessment of sexual maturation (mating, fertility) and neurobehavioral testing, particularly in rodents. For developmental neurotoxicity assessments, well-established methods should be used to monitor key central nervous system (CNS) functions, including assessments of reflex ontogeny, sensorimotor function, locomotor activity, reactivity, learning and memory.

Generally, biomarkers of adverse effects could be identified in non-clinical studies that should be useful in monitoring the human subjects in clinical trials. Prior to the initiation of trials in pediatric populations, results from repeat-dose toxicity studies of appropriate duration in adult animals, the core safety pharmacology package and the standard battery of genotoxicity tests should be

available. If a study is warranted, one relevant species, preferably rodent, is generally considered adequate.

The appropriateness of carcinogenicity testing should be addressed before long-term exposure in pediatric clinical trials. However, unless there is a cause for concern (e.g., evidence of genotoxicity in multiple tests, or concerns for procarcinogenic risk based on mechanistic considerations), carcinogenicity studies are not recommended to support the conduct of pediatric clinical trials.

## Immunotoxicity studies

Immunotoxicity studies are generally conducted for biologics and biotechnology-derived products (also classified as large molecules). Biotechnology-derived pharmaceuticals were initially developed in the early 1980s. Among biologics are the products derived from the characterized cells through the use of a variety of expression systems that include bacteria, yeast, insect, plant and mammalian cells. The intended use may include *in vivo* diagnostic, therapeutic or prophylactic uses. The active substances include proteins and peptides, their derivatives and products of which they are components; they could be derived from cell cultures or produced using recombinant plasma factors, fusion proteins, enzymes, receptors, hormones and monoclonal antibodies. Other products include recombinant DNA protein vaccines, chemically synthesized peptides, plasma-derived products, endogenous proteins extracted from human tissue and oligonucleotide drugs.

Regulatory standards for biotechnology-derived pharmaceuticals have been generally comparable among the three ICH regions namely European Union, Japan and United States and all three regions have adopted a flexible case-by-case, science-based approach to preclinical safety evaluation needed to support clinical development and marketing authorization. Per ICH guidances (S6, 1997 and S8, 2006), all new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity using standard toxicity studies, and additional immunotoxicity studies conducted as appropriate based on a weight-of-evidence review, including immune-related signals, should be completed before exposure of a large population of patients (e.g., Phase III). Immunogenicity may be related to either suppression or enhancement of immune response. Suppression can lead to decreased host resistance to infectious agents or tumor cells whereas the enhancement can exaggerate autoimmune diseases or hypersensitivity.

Immunosuppression or enhancement can be associated with two distinct groups: (1) drugs intended to modulate immune function for therapeutic purposes (e.g., to prevent organ transplant rejection) and (2) drugs not intended to affect immune function but cause

immunotoxicity due to necrosis or apoptosis of immune cells or interaction with cellular receptors (antiproliferative agents such as cancer drugs). Additional testing may be based on (1) findings from standard toxicity studies, (2) the pharmacological properties of the drug, (3) the intended patient population, (4) structural similarities to known immunomodulators, (5) the disposition of the drug and (6) clinical information.

Standard toxicity studies include (1) hematological changes such as leukocytopenia/leukocytosis, granulocytopenia/granulocytosis or lymphoma/lymphocytosis, (2) alterations in immune system organ weights and/or histology (e.g., changes in thymus, spleen, lymph nodes and/or bone marrow), (3) changes in serum globulin that occur without a plausible explanation, such as effects on the liver or kidney, which can be an indication that there are changes in serum immunoglobulins in the liver or kidney, and can be an indication that there are changes in serum immunoglobulins, (4) increased incidence of infections, and (5) increased occurrence of tumors can be viewed as a sign of immunosuppression in the absence of other plausible causes such as genotoxicity, hormonal effects or liver enzyme induction. Similar to the assessment of risk with toxicities in other organ systems, the assessment of immunotoxicity should include (1) statistical and biological significance of the changes, (2) severity of the effects, (3) dose-exposure relationship, (4) safety factor above the expected clinical dose, (5) treatment duration, (6) number of species and endpoints affected, (7) changes occurring due to stress, (8) possible cellular targets and/or mechanism of action, and (9) reversibility of effect(s). Additionally, T-cell-dependent antibody response (TDAR) and immunophenotyping of leukocyte populations can be conducted to identify the specific cell population.

For immunotoxicity studies, the choice of an animal species depends on the ability to develop an immune response to the vaccine antigen. Most human vaccines are immunogenic to rodents or rabbits. In some cases, only non-human primates may show an adequate immune response.

The measurement of antibodies associated with administration of these types of products is performed in repeat-dose toxicity studies and immune response (e.g., titer, number of responding animals, neutralizing or non-neutralizing) is correlated with any pharmacological and/or toxicological changes.

Some information on absorption, disposition and clearance in relevant animal models should be available prior to clinical studies that may provide margins of safety based upon exposure. Since the expected consequence of metabolism of biotechnology-derived pharmaceuticals is the degradation to small peptides and individual amino acids the classical biotransformation studies as performed for pharmaceuticals are not needed.

A generally accepted study design in rodents is a 28-day study with consecutive daily dosing but seldom using non-rodent species. Usually, both genders are used in these studies, excluding non-human primates, unless the intended use is in only one gender of the human population. Multiple dose levels are used with high doses above NOAEL dose, in order to determine dose-response relationships and the dose at which no immunotoxicity is observed.

DART studies are generally required for vaccines in order to determine the adverse effects on pregnant/lactating female animals and development and growth of the embryo/fetus and the offspring. In such studies, the females must be exposed to the vaccine during the interval from implantation through closure of the hard palate and also at later stages of pregnancy. The offspring should be followed to weaning and observed for growth and development. In addition, the immunological assessment may include the measurement of vaccine-induced antibody response to verify exposure to the embryo/fetus to maternal antibody.

### Phototoxicity studies

An evaluation of the non-clinical drug distribution to skin and eye should be completed to inform any human risk. An experimental evaluation (non-clinical *in vitro/in vivo* or clinical) of phototoxic potential should be undertaken before exposure of large numbers of subjects (Phase III). Alternatively, a direct assessment of phototoxic potential in a non-clinical or clinical study can be undertaken. If the phototoxicity assessment indicated a potential photocarcinogenic risk, the risk can usually be adequately managed in patients by protective measures including a warning statement in the informed consent for clinical trials and in product information for marketing.

### Abuse liability testing

Abuse potential refers to a drug that is used in non-medical situations, repeatedly or even sporadically, for the positive psychoactive effects ([Guidance for Industry FDA Abuse Potential, 2010](#)). The guidance for abuse potential of drugs is intended to assist with products that mainly affect the central nervous system and such drugs are regulated under the Controlled Substances Act (CSA; 21 U.S.C. 811, 2007). In addition, the drugs that are structurally and pharmacologically similar to other drugs with known potential of abuse such as antipsychotic drugs that may cause sedation, euphoria or mood changes are also regulated in the same way. The CSA has four schedules to categorize the substances, depending upon the schedules.

The purpose of scheduling substances under the CSA is to minimize abuse and diversion while affording appropriate therapeutic areas. The controls may include manufacturing and production quotas, varying degrees of manufacturing and distribution site requirements and import/export regulations. Prescribers, dispensers, drug manufacturers and distributors are required to register with the Drug Enforcement Administration (DEA).

Non-clinical data collected early in the drug development process can be useful in identification of early indicators of abuse potential that would typically be available before first human dose and include the PK/PD profile to identify the duration of action, similarity of chemical structure to known drugs of abuse, receptor binding profile and behavioral/clinical signs from *in vivo* non-clinical studies.

Three types of studies are often completed to evaluate the potential for abuse liability: (1) drug discrimination, (2) self-administration of the compound and (3) an assessment of withdrawal. When conducted, studies of drug discrimination and self-administration are generally stand-alone.

Among the types of animal abuse potential studies, self-administration tests assess the rewarding properties of a drug. Also, in the drug discrimination method, the animals indicate whether a test drug produces physical or psychotic perceptions similar to those produced by a known drug of abuse. In addition, the psychomotor tests assess the effects of the test drug on motor functioning in comparison with the effects of well-characterized drugs of abuse.

Pertinent data can be obtained during Phase III clinical trials that can provide support not only for therapeutic potential but also for abuse, dependence on potential drug diversion and accountability. Sometimes, abuse-relevant adverse event data for non-patient healthy populations can be obtained from single- and multiple-dose pharmacokinetic studies and electrocardiographic studies.

Any U.S. or foreign post-market experience and epidemiological data regarding misuse and abuse of a drug may be useful in decisions about scheduling a substance under the CSA and labeling a drug under the Food, Drug and Cosmetic Act (FDCA) of 1938. Labeling and drug scheduling play different roles in encouraging safe and appropriate use of drugs with abuse potential, as well as in minimizing the actual abuse, and the diversion. Information on abuse potential is generally conveyed to the health care professionals and patients through appropriate labeling which is the cornerstone of risk minimization for most of the approved drugs.

## Impurities qualification

The approaches for qualifying impurities and degradants are outlined in ICH guidances ([Guidance for Industry](#)

[Q3A \(R\), 2008](#) and [Q3B \(R2\), 2006](#)). An impurity can occur in a new drug either as a result of chemical synthesis or the byproduct of degradation process. Impurities can be classified into (1) organic impurities (process and drug related), (2) inorganic impurities and (3) residual solvents. Organic impurities can arise during the manufacturing process and/or storage of the new drug substance. Inorganic impurities can result from the manufacturing process. They are normally known and identified and may include: (1) reagents, ligands and catalysts, (2) heavy metals or other residual metals, (3) inorganic salts and (4) other materials (e.g., filter aids, charcoal).

Solvents are inorganic or organic liquid used as vehicles for the preparation of solutions or suspensions in the synthesis of a new drug substance. Others may include extraneous contaminants that should not occur in new drug substances and are more appropriately addressed as good manufacturing practice (GMP) issues. The actual and potential impurities most likely to arise during the synthesis, purification and storage of a new drug substance should be described. The summary should include test results of batches manufactured during the development process and batches from the proposed commercial process, as well as the results of stress, used to identify potential impurities arising during storage. Any degradation product observed in stability studies at the recommended storage conditions at a level greater than the identification threshold should be identified. When identification of an impurity is not feasible, a summary of the laboratory studies demonstrating the unsuccessful effort should be included in the regulatory application.

The acceptance criteria of impurities should be based on Pharmacopoeial standards (U.S. Pharmacopoeia, European Pharmacopoeia and Japanese Pharmacopoeia) or any other known safety data. Analytical results should be provided in an application for all batches of a new drug substance used for clinical, safety and stability testing, as well as for batches representative of the proposed commercial process.

Qualification of impurities is the process of acquiring and evaluating data that establish the biological safety of an individual impurity or a given impurity profile at the level(s) specified. The level of any impurity present in a new drug substance that has been adequately tested in safety and/or clinical studies is considered qualified. Impurities that are also significant metabolites present in animal and/or human studies are generally considered qualified. In the absence of supporting safety data, genotoxicity tests along with acute and subchronic toxicity studies (based upon the intended use of the product) are conducted to demonstrate that the impurities at specific levels would not pose any hazards.

The reporting, identification and qualification thresholds for impurities are recommended in these guidances, based upon maximum daily dose of the drug. *For the*



*purpose of this chapter, only the dose less than 2g is referred.* For maximum daily dose of drug less than 2g (1) the identification threshold should be 0.1% or 2mg whichever is lower and (2) the qualification threshold should be 0.15% or 3mg whichever is lower.

The qualification of biological impurities such as peptides, oligonucleotides, radiopharmaceuticals, fermented and semi-synthetic products, herbal products and crude products of animal or plant origin are not covered under Q3A and Q3B guidelines. In the case of biologics, potential risks may be associated with host-cell contaminant derived from bacteria, yeast, insect, plant and mammalian cells that can result in allergic reactions and other immunological effects. For products derived from insect, plant and mammalian cells or transgenic plants and animals, there may be an additional risk of viral infections.

## ESTIMATION OF THE FIRST DOSE IN HUMANS

Extensive non-clinical data collected from a large number of acute and repeat-dose toxicity studies help determine the initial safe and maximum recommended starting dose (MSRD) and dose range for the Phase I human trials using healthy subjects (80–100) and identify any parameters for clinical monitoring for potential adverse effects (Guidance for industry, maximum safe starting human dose, July 2005). All the relevant preclinical data including pharmacologically active dose, the full toxicological profile and the PK/TK and ADME data of the therapeutic agent are considered when determining MSRD in the algorithmic process.

In calculation of MSRD, NOAEL is the major element tested in animal species. In order for conversion of NOAEL to human equivalent dose (HED), a species-specific conversion factor (conversion factor table described in the guidelines) is used. Although only NOAEL is used in the algorithm for calculating an MSRD, other data (exposure/toxicity, relationship, pharmacological data or prior clinical experience with related drugs) can affect the choice of most appropriate species, scaling and safety factors. The conversion should be based on the normalization of the doses to body surface area. The extrapolation of animal dose to human dose is done by dividing the NOAEL in each species by appropriate body surface area conversion factor (BSA-CF). This conversion factor is a unitless number that converts mg/kg dose of each animal species to the mg/kg dose in humans which is equal to the animal's NOAEL in mg/m<sup>2</sup> basis. The resulting value is HED and the species that generates

the lowest HED is considered the most sensitive species. The formula for HED calculation is shown below:

$$\text{HED} = \text{animal dose in mg/kg} \times (\text{animal weight in kg/human weight in kg})^{0.33}$$

In order to calculate MSRD, a safety factor (usually 10) is applied to HED in order to increase the assurance that the first dose in humans will not cause any adverse effects. However, it can be raised when there is reason for increased concern and lowered when concern is reduced because of available safe data.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

It is recognized that significant advances in harmonization of the timing of non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals have already been achieved and are detailed in the referenced guidances in this chapter, and continued efforts are ongoing to maintain harmonization for pharmaceutical development globally. However, some differences still remain in a few areas. Regulators and industry will continue to consider these differences and work towards further improving the drug development process. In the last few years, a number of computation toxicology models have been developed (e.g., *in silico modeling*) as alternatives to *in vitro* and *in vivo* models and in efforts to reduce animal testing; however, computational testing has not been approved by regulators for pharmaceutical development at this time. Recently, FDA has developed an Informatics and Computational Safety Analysis program for future archiving of safety data, but at this time it is still a work in progress.

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# Statistics in veterinary toxicology

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## INTRODUCTION

Veterinary toxicology has seen a dramatic and consistent increase in available statistical resources in the past few decades. Currently, free resources locatable by web searches include formulae and examples for common statistical techniques, online or freely downloadable software to perform many common biostatistical analyses, sample data sets, statistical guides from governmental and private organizations and biostatistics course notes from multiple universities. What is not necessarily clear from any of the resources above or from many of the similar for-pay resources is that there is a framework or process for conducting or facilitating a quality statistical analysis. The more complete process is:

- 1 Define the objective of the analysis, i.e., "what is the question?"\*
- 2 Determine the types of data.\*
- 3 Identify constraints/limitations on the study data and analytical methods.\*
- 4 Generate preliminary graphs and summary statistics of the data.\*
- 5 Choose the correct technique based on items 1–4 above.
- 6 Perform the analysis.
- 7 Interpret the results and generate graphs and summary statistics of the significant results.

\*These steps are not strictly ordered.

All of these, or at least how they will be addressed, should be considered before a study is conducted. Following the complete procedure will increase understanding of the data and lessen the chance of making

mistakes. Unfortunately, detailed coverage of these steps would fill at least one and probably several books. A veterinary toxicologist should be able to perform some simple analyses based on this chapter alone, but our main purpose here is to provide an overview of the process with enough detail so that an investigator can evaluate and utilize other resources as needed, application of the approach are addressed in Gad (2008).

## Core concepts and vocabulary

### *Relationship between variables*

One of the most important concepts to understand is that statistics can never actually prove or disprove causation. Correlation, a statistical measurement of how strongly variables are related, is not causation no matter how strong the correlation is.<sup>1</sup> Statistics can instead show, to a reasonable and often definable degree of confidence, whether or not one or more variables are *related to*, *associated with*, or *correlated with* one or more other variables. The variable for the outcome of interest is called the dependent variable, while the other variables are called independent variables, a.k.a. predictor or explanatory variables. Variables that the investigator has control over are automatically independent variables. For example, independent variables for an antibacterial drug study could be animal species, gender, age, number

<sup>1</sup>Toxicologists who want a short introduction to causation reasoning should start with Austin Bradford Hill's classic paper "The environment and disease: association or causation?" (Hill, 1965).

per dose group and drug dosage. Measurements such as food consumption, animal weight, clinical observations, plasma bacterial counts and blood chemistry and hematology would be dependent variables in this example. The independent versus dependent distinction may seem blurry for retrospective analyses or data mining, where the investigator is picking data to analyze rather than reporting on an experiment. In such cases, decide on the outcome of interest first, and then the variables that measure the outcome of interest are the dependent variables while all other available data constitute the independent variables.

### Statistical significance and statistical error

When a finding is statistically significant at a probability of 0.05 or less, usually written as  $p \leq 0.05$ , the probability of random chance producing either the data seen or any more extreme data is 0.05, or 5%. The choice of a 95% level of confidence for declaring statistical significance is arbitrary but has immense historical inertia and regulatory enshrinement and therefore  $p \leq 0.05$  should be considered the default choice. Unfortunately, statistical significance is not always correlated with the existence of a real effect, as shown in Table 12.1.

A Type I error is called a false positive and the probability of a Type I error is indicated by  $\alpha$  (alpha). A Type II error is called a false negative but instead of having a symbol specifically for Type II error probability,  $\beta$  (beta) is used to indicate one minus the chance of a Type II error:  $\beta = 1 - \text{probability (Type II error)}$ . Beta is called power and can be read as the chance of finding no effect if there really is no effect. It should be clear that neither error is desirable. Fortunately, the two types of error can be controlled in a statistical analysis but there is an unavoidable tradeoff: as  $\alpha$  is decreased (less chance of a false positive) and  $\beta$  is increased (less chance of a false negative), the number of animals needed to find a specific effect size is also increased. Put simply, if the investigator wants less chance of making statistical errors or wants to discern smaller effect sizes, they will need to use more animals or other experimental units (such as cell culture units). The exact relationship between  $\alpha$ ,  $\beta$ , effect size and animal counts is different for differing statistical analyses, but good tools for calculating these exist online and in many software packages and textbooks. When in doubt, eqn (1) should be used as an estimator for interval dependent data.

$$n \geq 2s^2 \frac{(Z_{\alpha/2} + Z_{\beta})^2}{\Delta^2} \quad (1)$$

where:

- $n$  = number of required samples or animals in each test arm (roundup).
- $s$  = the expected standard deviation of the sample data.
- $Z_{\alpha/2}$  = the Z-statistic for the desired alpha. Technically, this is the value at which the cumulative standard normal distribution reaches  $1 - \alpha/2$ . Practically, since alpha is almost always 0.05, this value is usually 1.96.
- $Z_{\beta}$  = the Z-statistic for the desired beta. Most calculations use  $\beta = 0.80$  ( $Z_{\beta} = 0.842$ ) or  $\beta = 0.90$  ( $Z_{\beta} = 1.282$ ).
- $\Delta$  = the expected difference in effect size between groups.

This can be read as “In order to find a difference of  $\Delta$  or more between groups, with a false-positive rate of no more than  $\alpha$  and a false-negative rate no more than  $1 - \beta$ , and assuming the sample standard deviation will be no more than  $s$ , then at least  $n$  animals per group are required.” Since both  $s$  and  $\Delta$  in the equation are squared,  $n$  inflates rapidly as standard deviation increases or the desired effect criterion (difference) decreases.

The question of the relevance or meaning of statistical significance is also complicated by considerations of biological significance. Biological significance generally means a change or condition that could appreciably affect the health of an animal (in humans, “clinical significance”). To understand how the significances can be distinct, consider a study where each animal has a number of blood samples drawn over a period of time but care is taken that the total volume of blood taken will not significantly affect animal health. Because of the repeated blood sampling there are real decreases in the red blood cell-related parameters such as hemoglobin and hematocrit and the changes are consistent enough that statistical analysis of the time trends will usually find these changes. However, neither the real hematological changes nor the statistically significant findings mean the changes are biologically significant. Therefore, a statistic is not a substitute for sound professional judgment but in order to make intelligent conclusions veterinary toxicologists should be aware of the difference between the existence of a real effect, finding such an effect statistically and the biological significance of an effect.

TABLE 12.1 Possible types of statistical error

		Was the result statistically significant?	
		No	Yes
Is there a real effect?	No	No error	Type I error
	Yes	Type II error	No error

### Degrees of freedom

Most statistical methods produce or refer to a measure called degrees of freedom (df). The degrees of freedom are the number of levels of each independent variable that are not fixed by the calculation. The usual



illustration is that if there are  $N$  observations and the mean of the observations is known, then  $N - 1$  of the observations can be anything but one of the observations is constrained because the means formula has to come out to the known mean. The degrees of freedom in the example is therefore  $N - 1$  because that is the number of non-fixed or free observations. Degrees of freedom is a very important statistical concept, but practically, it is not an intuitive concept for most people and software will normally handle the df calculations without intervention so there is no pressing reason to understand the concept unless the toxicologist is studying statistics.

## OBJECTIVE OF THE ANALYSIS

Statistical methods generally perform some combination of three functions:

- 1 The most commonly employed function is hypothesis testing. Examples of hypothesis testing include determining if groups of data are different from each other or deciding if an agent has a significant effect on a specified outcome.
- 2 A second function is building statistical models which may then be used for predicting the outcomes of experiments not previously performed or improving disease diagnosis and treatment. A statistical model is simply a set of mathematically defined relationships between the treatment and response aspects (variables) in the study that show both the types of relationships, e.g., linear, and the strengths and best estimates of the relationships.
- 3 The third function, reduction of dimensionality, has the goal of reducing the number of variables in a statistical model without significant reduction of information. Less complex models are desirable because they are easier to understand and make predictions from. The production of descriptive or summary statistics is the second most commonly utilized statistical function in toxicology and can be considered a subset of reduction of dimensionality. In general most descriptive statistics are some measure of central tendency, such as a mean or median, plus some measure of variability, such as standard deviation or range (minimum and maximum). The inclusion of summary statistics as a subset of reduction of dimensionality is common in toxicology literature but statistical literature considers these two separate functions and usually refers to reduction of dimensionality as dimension reduction.

## TYPES OF DATA

The types and quantities of available data limit what statistical techniques are valid so it is important to characterize both the independent and the dependent variables. Variables can in general be one of:

- Interval, a.k.a. continuous: the variable can take on any value within a range of numeric values. Examples include body weights or blood chemistry readings.
- Categorical: the variable can only take on a limited number of values and the categories have no intrinsic order. Examples include gender or species.
- Ordinal, a.k.a. ranked: the variable can take on a limited number of values but there is some intrinsic order to the values. Examples include growth stage for male horses (foal, colt or stallion) or the Draize scale for irritation ([Draize, 1959](#)).

One important subtype of variable is the binary variable, i.e., a variable that can take only one of two values. Gender is the classic binary variable in biological sciences. Both categorical and ordinal variables that can only take two values should be analyzed as binary variables. Therefore, all independent and dependent variables should be classified as one of interval, binary, categorical or ordinal. When deciding on variable classification, the investigator must take care to distinguish between what values are possible for a variable and how they are measured. For example, imagine a study that classified animals as underweight, nominal weight or overweight but did not record the actual weights. Normally body weight is an interval variable but in that case, it would be an ordinal variable because the only measure of body weight is one of three ranked classes.

## Changing the data

Occasionally data need to be changed before analysis. Common types of changes include coding ordinal values, imputing values, transformations and removal of outliers.

### *Coding ordinal variables*

Because of their nature, ordinal variables often have to be renamed before analysis because both the value of the variable and the order in relationship to other values has to be evaluated correctly. In the example for growth stages for male horses above, a veterinarian would recognize the inherent order foal→colt→stallion but a machine would alphabetize the terms and order them as colt→foal→stallion. Therefore ordinal variables are

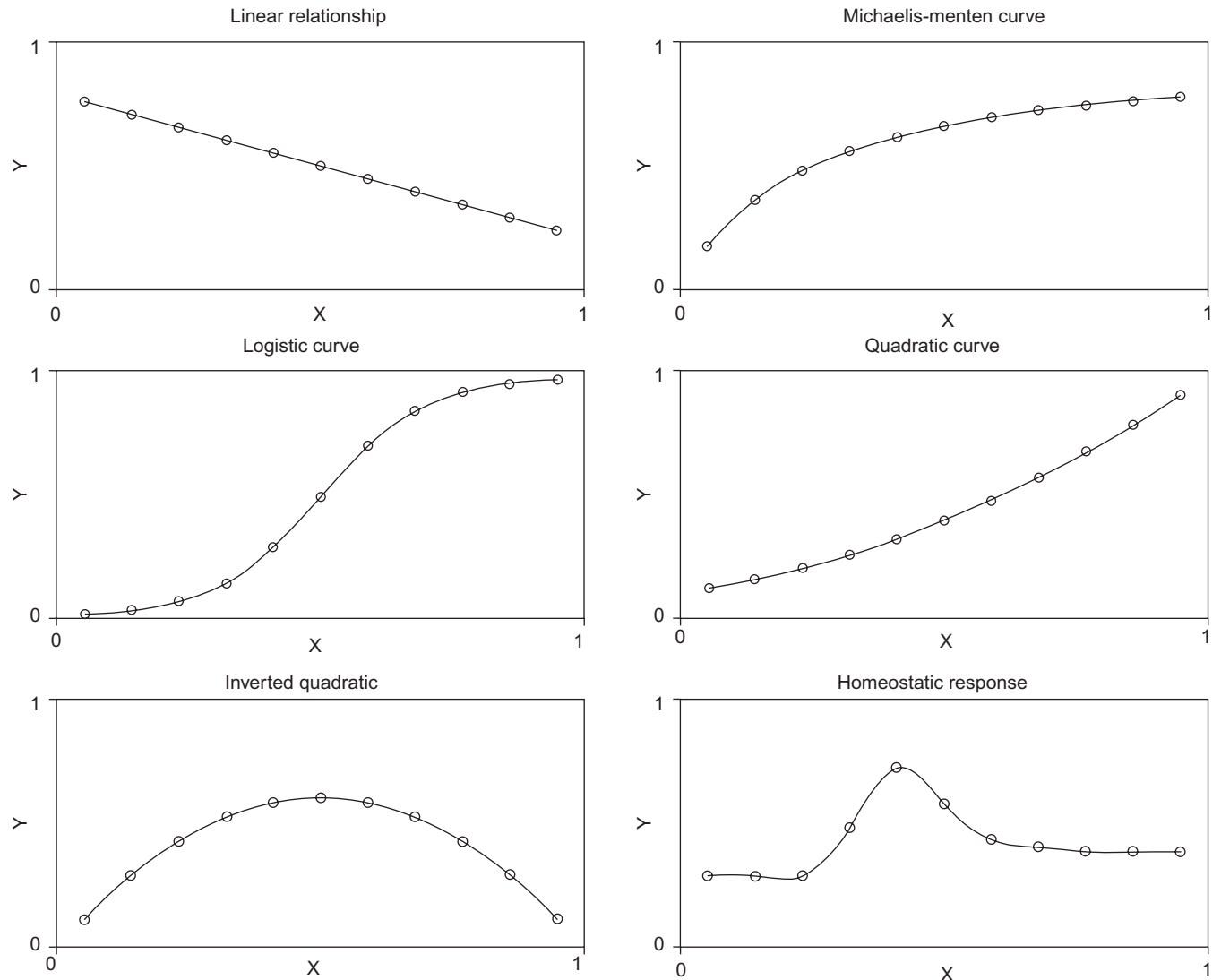


FIGURE 12.1 Idealized relationships between independent and dependent interval variables.

often renamed to either the series 1, 2, 3...or the series A, B, C...before analysis so that the software automatically recognizes the proper order. This renaming is called coding.

### Imputation

Statistically speaking, imputation is the assignment of values to censored observations. There is some historical precedent for imputation but the practice has never been statistically valid and is not recommended.

### Transformations

As seen in Figure 12.1, the relationship between the independent and dependent variables can be many shapes besides linear and, indeed, truly linear relationships

between variables over their full range of possible values are vanishingly rare. Therefore, it is often tempting to transform the data before analysis to make the relationship linear, thereby making both the calculations and the interpretation easier. The necessity of transformation before analysis has decreased over time because valid statistical techniques for a variety of variable relationships have become more available, but there is still both some historical impetus towards linearizing data and the occasional data set for which transformation is desirable. Generally, transforming data is rarely necessary for valid analysis, but may be reasonable if (1) there is a valid statistical, regulatory or other reason to use a transformation rather than a different statistical technique on untransformed data and (2) the transformation method and criteria for using it is decided before the data are analyzed further.

Common transformations circumstances in toxicology include:

- In cases where repeated samples are taken from an animal over time it is common practice to subtract the baseline value or mean of the baseline values from post-exposure samples so that results can be read as change from baseline. This is a reasonable transformation, but there are minor statistical objections and modern analysis methods generally make this unnecessary.
- For studies that dose by absolute dose amount, transforming drug dose to dose per body mass, e.g., replacing  $\text{mg}_{\text{drug}}$  with  $\text{mg}_{\text{drug}}/\text{kg}_{\text{body\_mass}}$  as an independent variable, is recommended. In virtually all cases, individual dose per body mass will fit models closer than absolute dose.
- A valid statistical reason to transform data is heteroscedasticity, meaning the variability of the dependent variable is not the same for each observation. Most statistical models assume that variance (amount of variation) is the same for all observations but, realistically, variance for many biological measurements increases with the magnitude of the measured value. This increase is known as variance inflation. Figure 12.3 illustrates heteroscedastic variance inflation in that the range of values increases significantly from group A to group C. If the investigator knows or suspects that heteroscedasticity exists, two good general methods for variance stabilization are Tukey's Ladder of Powers (Tukey, 1977) and the Box-Cox transformations (Box and Cox, 1964). The choice of which to use is primarily based on which is available in the analysis software. Both transformations are intended for the case of a single dependent interval variable where the magnitude of the variance is roughly proportional to the magnitude of the dependent variable.
- One common transformation example arises from pharmacokinetic calculations, where both area under curve (AUC) and maximum observed value ( $C_{\text{max}}$ ) values for the majority of species and studies are approximately lognormal distributed, which is visible when graphed (see Figure 12.6) and also leads to variance inflation in higher parameter values. Therefore it is recommended to take the logarithm of AUC and  $C_{\text{max}}$  values before analysis.

### Outliers

The term outlier applies to individual data points that are well outside the expected range of the data and usually only applies to dependent variable values. Outliers can occur in categorical or ordinal data but the term is usually applied to interval data. Under the assumption that interval data are normally distributed, i.e., follow the classic bell-shaped curve, about 68% of the values

will be within one standard deviation from the mean, about 95% of the values will be within two standard deviations and about 99.7% of the values will be within three standard deviations. Many box plots will indicate outliers as single points outside of the contiguous boxes and lines and this is the reason box plots may be preferable over scatter plots. Outliers are undesirable because they increase calculated variability disproportionately and may bias estimates of means. However, removal of outliers is only valid if the outliers are clearly documented experimental errors.

## IDENTIFYING CONSTRAINTS

Even after the types and quantities of data have been identified there still may be more than one appropriate statistical analysis. Other considerations can limit the list of reasonable or possible analysis methods and can also change the interpretation of statistical results, even if there is only one sensible statistical technique. Therefore, identifying other possible restrictions should be a fundamental part of any analysis. Common constraints for toxicology analyses and reasonable responses to the constraints are as follows:

**Constraint:** Biological, financial, temporal, logistical, and other practical realities limit the amount and types of data that can be taken. For example, it is not reasonable to get multiple closely spaced blood samples from small animals (biological limitation), to get a large number of organ samples from large animal species (financial limitation), to reliably estimate background tumor incidence in any species in less than the majority of the species' lifetime (temporal limitation) or to use sufficient numbers of animals of exactly the same age and source to power a chronic toxicity study as one would a Phase III clinical study.

**Response:** Smart study design can mitigate many of these problems. Practically, this means using designs that are already well tested and well understood. Such designs can be found in toxicology literature and other publications and established toxicology labs will have outlines for such studies. Operational limitations also require careful interpretation and the investigator should use professional judgment when deciding how generally or specifically the conclusions apply.

**Constraint:** Data sets are often small, from heterogeneous populations, inconsistently measured, or not necessarily from the population of interest. These factors are grouped together because they interact, so a designed study controls population homogeneity and measurement consistency, but is often small due

to financial or other operational constraints. Also, designed studies may not be reasonable to perform in the population of interest. On the other hand, research and data mining may yield larger data sets from the population of interest at the price of increased uncertainty in measured values.

**Response:** This is usually an interpretation problem, as noted above. If the investigator wants to analyze results across multiple studies or from multiple data sources then they should explore the field of meta-analysis.

**Constraint:** Regulatory agencies may have preferred statistical methods for certain studies. Similarly, professional organizations or publications may have favored statistical methods due to professional consensus or even historical inertia.

**Response:** The method suggested by the regulatory or professional organizations should be used unless there is compelling evidence that the suggested analysis would be invalid.

**Constraint:** Most commonly the investigator is examining a minutely small fraction of the animal population, but with endangered species, rare breeds and similar cases it may be possible to collect data for a significant fraction of the species of interest.

**Response:** In the latter case, there are more accurate methods to calculate some summary statistics and to perform some statistical analyses, but the methods are different than those presented in beginning biostatistics courses. There is nothing wrong with using standard statistics, but if the investigator wants increased precision then they should examine sampling statistics.

**Constraint:** Study design for designed studies, or data collection design for data mining, can determine which statistics are valid. For example, relative risk, a.k.a. risk ratio, is not a valid statistic for case-control studies and many retrospective studies on comparative incidence rates, even though it is technically calculable. Odds ratios are valid in such cases.

**Response:** Relative risk is the most common biostatistic that can be invalidated because of study design. In general, if the investigator wants to model or perform hypothesis tests about comparative incidence rates, relative risks or similar topics, then they should learn more about categorical data analysis, which includes analysis of such data.

**Constraint:** If both the dependent variable and the independent variables are all binary, ordinal or categorical, then the amount of data can be a deciding factor in the choice of analysis. This happens because many analysis methods for such data are based on approximations that only become valid for a sufficient volume of data.

**Response:** Count the number of animals or observations for each unique combination of the variables. Tables are the best way to do this. The cutoffs where different methods should be used depend on the statistical test

and are therefore covered in the section on exact tests and chi-square tests below. Alternatively, the investigator can group categories together to get sufficient numbers per category.

**Constraint:** Data are often censored by circumstances other than an investigator's design. In this case, censoring means not all the data could be collected as desired. Common censoring circumstances include animal death or debilitation, spoiled samples such as coagulated blood samples, skipped experimental procedures or, in the case of retrospective analysis, imperfect access to records.

**Response:** If data were censored at random then censoring is not a serious limitation because statistical techniques that work for censored or unbalanced data have been developed. "At random" in this case means that the pattern of data loss has no relationship to the values of the independent or dependent variables. However, if the censoring is related to the values of other variables, then inference on those variables is potentially biased after the censoring occurs. In the latter case, the only statistically analyzable dependent variable is time to censoring.

Limitations caused by censoring deserve some additional explanation. Consider a group of animals given a substance that severely inhibits red blood cell (RBC) production. The animals with dangerously low RBC values will die or be taken off study *in extremis*. Therefore, group mean RBC values are only representative of dosed animals *before* any animals are taken out of the study. Any statistics afterward are biased because the worst case(s) were eliminated from consideration. Therefore RBC values cannot be used as a dependent variable because they are inherently biased and the amount of the bias cannot even be reliably estimated. If there are enough deaths or removals *in extremis*, then the times to removal can be analyzed as the dependent variable and the relationship between the independent variables and time to removal can be estimated. However, there is a broad gap between theory and practice. The statistical theory says that even one death that may be related to the effects being studied makes time to death the only valid dependent variable, but if that criterion were applied rigorously, then a large proportion of studies would be unanalyzable. More practical guidelines are:

- All censoring must be clearly documented.
- Possible reasons for censoring must be investigated and also documented.
- If the censoring has no relationship or no clear relationship to the values of the dependent or independent variables then proceed with analyses as planned.
- If the censoring has a clear relationship to other variables' values then time-to-censoring is the only valid



dependent variable for the whole study but the portion of the study before censoring occurs may be analyzed as planned as long as the demarcation is clearly documented. Proceeding with the planned analysis but reporting that censoring may have biased the results is also common and not unreasonable as long as the possible bias and reason for it is reported in all study results.

Time to death analysis, also called survival analysis, is a subset of time-to-event analysis. One common category of toxicology studies that includes both survival analysis and time-to-event analysis is carcinogenicity studies.

## GRAPHING AND SUMMARIZING DATA

Examining data graphically *before* analysis is often underemphasized and all too rarely employed. To understand why this might be significant, consider that one of the best analysis tools where the dependent variable is interval data and the independent variables are mixed interval, binary and/or categorical data is Analysis of Variance (ANOVA). Now assume that the actual data are fairly close to the idealized relationships shown in [Figure 12.1](#). Which relationships will ANOVA produce correct results for? The answer is: only the linear relationship. ANOVA will probably find the first quadratic, the Michaelis-Menten curve and the logistic relationships to be statistically significant, but will estimate the best fitting straight line, entirely missing the curvature. ANOVA will estimate no relationship between the independent and dependent variable (flat line) for the inverted quadratic relationship and may or may not find significance for the homeostatic curve despite the existence of strong relationships. Therefore, if the analyst does not graph the variables before analysis, then serious errors can be made even when using a powerful, well-accepted technique. If the analyst does graph the relationship first, then inclusion of a single quadratic term will produce a valid analysis of both the linear and quadratic cases and closely approximate the Michaelis-Menten curve. The other two cases require more advanced techniques. Note that only the inverted quadratic relationship is unlikely because close approximations of all the others can be found in toxicologic data.

The suggested graphs for various types of variables are shown in [Table 12.2](#). These are by no means the only possible graphs but are well accepted and unlikely to lead to serious misrepresentation of the data. Bar graphs are often referred to as bar charts and boxplots may be called box and whisker plots or just box plots. [Figures 12.2–12.4](#) show an example bar graph, boxplot

and scatterplot, respectively. Other example scatter plots are shown in [Figure 12.5](#). Toxicologists who want to further explore good data presentation techniques could start with Edward Tufte's classic *The Visual Display of Quantitative Information* ([Tufte, 1983](#)).

Along with the generation of initial graphs, some summary statistics for both the dependent and independent variables should be generated before analysis. Two general guidelines for summary statistics are:

- Interval variables should generally be summarized by mean, standard deviation, range (minimum and maximum) and count (number of animals or observations). Interval variables that are far from normally distributed should be summarized by median, quartiles (values that identify the boundary for the lowest and highest quarters of the data), range and count.
- Binary, categorical or ordinal variables should be summarized by tables of how many animals or observations fall into each unique combination of variables.

Initial summary statistics are primarily supposed to be an aid to understanding and another way of avoiding analysis errors. Therefore, initial summaries should use a reasonable minimum of statistical calculations and should be presented in a clear format. Example tables can be found in professional publications and many organizations will have example reports with tables or templates for generation of summary statistics.

Survival and time-to-event analyses use a life table to summarize data. An example life table is shown in [Table 12.3](#). The failures column indicates the number of animals developing the disease or condition of interest during the given time interval. The censored column shows the number of animals who died or were removed from the study and is unrelated to the number failing. Conditional failure probability is calculated as  $(1 - \text{failures}/(\text{at risk}))$  for each age category and cumulative failure is the cumulative multiplication of the conditional failure. The conditional and cumulative failure avoid counting censored animals, so, for example, even though over 50% of the population is gone by age 65–70, the failure rate does not go over 50% until age 70–75. Plotting the cumulative failure in a stepwise fashion and adding markers for the censored animals results in a Kaplan-Meier graph ([Kaplan and Meier, 1958](#)), which is the fundamental graphical tool for survival analysis.

Once the graphs and summary statistics are generated, the investigator should examine variables for differences related to a single variable, usually called main effects, and differences related to pairs of variables, usually called interactions. Common main effects unrelated to treatment are: animal weights vary by gender, breed and life stage; and normal hematology, blood chemistry and urinalysis values are somewhat different by gender and breed.

TABLE 12.2 Suggested graphs based on data types

Dependent variable(s) <sup>1</sup>	Independent variable(s) <sup>1</sup>				
	Single variable			Multiple variables	
	Continuous	Binary	Categorical or ordinal	Continuous	One or more continuous plus one binary, categorical, or ordinal
<b>Single variable</b>					
Continuous	Scatterplot	Scatterplot or boxplot <sup>2</sup>	Scatterplot or boxplot <sup>2</sup>	Separate graphs <sup>3</sup>	Scatterplot of means <sup>4</sup>
Binary	Scatterplot or boxplot <sup>5</sup>	None <sup>6</sup>	Bar graph or none <sup>7</sup>	Separate graphs <sup>3</sup>	Boxplot <sup>8</sup>
Categorical or ordinal	Scatterplot or boxplot <sup>5</sup>	Bar graph or none <sup>7</sup>	Bar graph or none <sup>7</sup>	Separate graphs <sup>3</sup>	Boxplot <sup>8</sup>
<b>Multiple variables</b>					
Continuous	Scatterplot <sup>9</sup>	Boxplot <sup>9,10</sup>	Boxplot <sup>9,10</sup>	Separate graphs <sup>11</sup>	Separate graphs <sup>11</sup>

## Notes

<sup>1</sup>Independent variables are graphed on the X (horizontal) axis and dependent variables are graphed on the Y (vertical) axis. In variable combinations other than those listed, first separate the variables so that they fit one of the existing categories.

<sup>2</sup>Vertical boxplots, each box represents a single level of the independent variable.

<sup>3</sup>Separate by the independent variables and graph accordingly.

<sup>4</sup>Separate means for each level of the binary, categorical or ordinal variable, connected by lines. Consider putting in error bars.

<sup>5</sup>Horizontal boxplots, each box represents a single level of the dependent variable. For scatterplots, point markers identify levels of the dependent variable.

<sup>6</sup>This case can be clearly represented by a table; a bar graph rarely adds significant value.

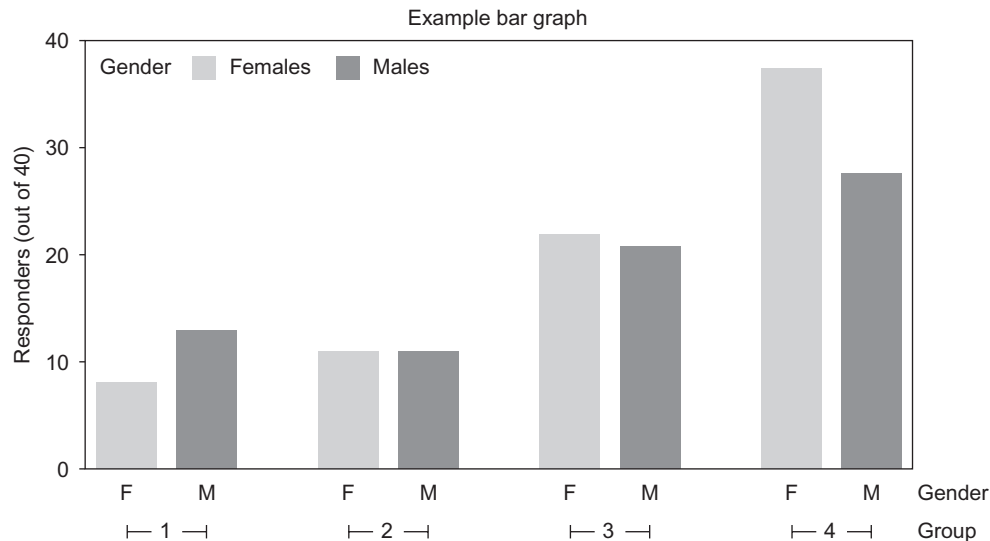
<sup>7</sup>Tables are also illustrative in this case. In general, relatively small values should use tables; larger values should use bar graphs. For bar graphs, both independent and dependent variables show on the X-axis, grouped by the dependent variable, and the Y-axis represents counts for each category.

<sup>8</sup>Separate horizontal boxplots for each unique combination of independent and dependent binary, categorical and ordinal value, grouped by the dependent variable value.

<sup>9</sup>If the dependent values are on the same scale, a single graph is possible using different point markers to identify separate dependent variables. Otherwise use separate graphs for each dependent variable.

<sup>10</sup>Vertical boxplots, each box represents a single independent variable.

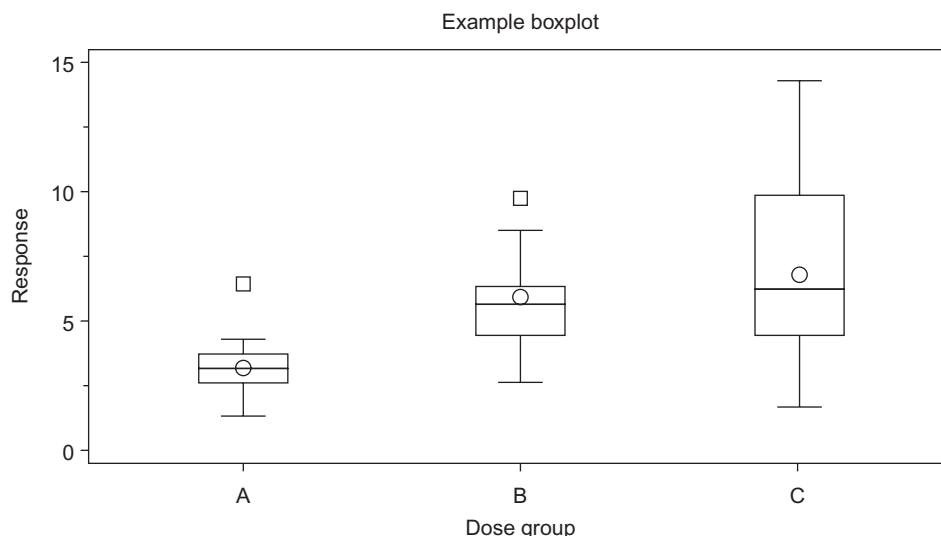
<sup>11</sup>Separate by both the dependent and independent variables until the case matches a group listed here and graph accordingly.



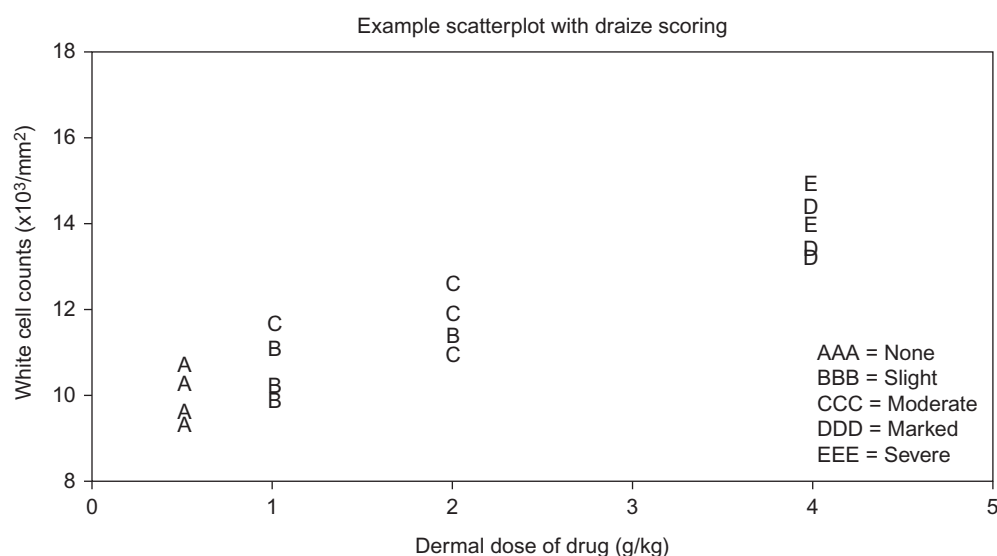
**FIGURE 12.2** A sample bar graph illustrating the number of responders grouped by the ordinal variable group and the binary variable gender. Potentially useful variations include showing percentage of responders instead of numbers or stacking the bars to show proportions of a whole.

Common main effects related to increasing drug exposure, called the three dimensions of dose response, are: increased proportion of responders, increased severity of response and decreased time to response and/or faster response progression. For graphs of an independent variable versus a dependent interval variable, examine the shape of the relationship and refer to [Figure 12.1](#) and

the discussion on it. Approximately linear relationships can be well modeled by the techniques discussed here, shapes with curvature in only one direction can usually be well modeled by including a quadratic term in the model, S-shaped curves can be modeled using logistic regression, but shapes with complex curvature require more advanced techniques.



**FIGURE 12.3** Boxplots are relatively information dense. The box extends to the lower and upper quartiles (the values which 25% and 75% of the data fall below, respectively). The capped lines extend to the minimum and maximum in some plots but for this one they extend to the 5% and 95% percentiles (the values which 5% and 95% of the data fall below, respectively). The line in the box is the median and the circle in the box is the mean, while the squares outside of the boxes represent outliers (values far enough from the mean or median to be considered unusual). Note that capped lines in other graphs indicate the standard deviation of the data rather than the range.



**FIGURE 12.4** An example scatterplot showing white cell count in relation to drug dose, with the muscle irritation score as the point symbol.

An interaction means the relationship between one independent variable and the dependent variable depends on the value of another independent variable. Common interactions in toxicology include: the shape of a dose-response curve may depend on gender, tumor incidence increases dramatically with both carcinogen exposure and the presence of oncogenes, and drugs that compete for binding sites have non-additive effects when coadministered. Both metabolic induction and inhibition can also produce interaction effects and the

timing of the interaction depends on how fast induction or inhibition happens. When interaction appears to be present, an interaction term should be put into the statistical model. Figure 12.5 shows the difference between no effect, a main effect and a main effect with interaction. Interactions without main effects are less common but if an interaction is present then the main effects must remain in the model.

Graphing and summarizing the data before analysis is in conflict with pure statistical theory. One of the

TABLE 12.3 Example life table

Animal age (months)	# at risk	Failed	Censored	Conditional failure probability	1-(Cumulative failure estimate)
00-01	100	2	1	0.9800	0.9800
01-05	97	1	0	0.9897	0.9699
05-10	96	0	0	1.0000	0.9699
10-15	96	1	0	0.9896	0.9598
15-20	95	1	0	0.9895	0.9497
20-25	94	1	0	0.9894	0.9396
25-30	93	0	1	1.0000	0.9396
30-35	92	1	2	0.9891	0.9294
35-40	89	3	1	0.9663	0.8980
40-45	85	3	2	0.9647	0.8664
45-50	80	5	3	0.9375	0.8122
50-55	72	5	5	0.9306	0.7558
55-60	62	2	9	0.9677	0.7314
60-65	51	0	13	1.0000	0.7314
65-70	38	8	6	0.7895	0.5774
70-75	24	12	0	0.5000	0.2887
75-80	12	1	6	0.9167	0.2647
80+	5	2	3	0.6000	0.1588

absolute rules of statistics is that you cannot change the analysis methods or goals just because you do not like the first answer. However, there is often more than one valid analysis method and there is no guarantee that two or more valid methods will produce the same results, so choice of method can influence results. Even single analysis methods usually have details that can be tweaked in various ways that may alter the results. Since an experienced analyst can at least guess at some answers by looking at graphs or even at raw data, this implies that analysis methods should be set before seeing *any* of the data, because otherwise a biased analyst could pick the analysis method or method details based on the outcome they wanted to show. The ideal of setting the exact analysis before reviewing the data should be the goal for all statistical analyses and is approachable for well-controlled studies with experienced investigators and analysts but practical experience suggests that newer investigators will make more and worse mistakes by not doing a preliminary examination of the data first. The controls that should be put in place and followed rigorously are:

- The overall goal (modeling or hypothesis testing) must be decided first.
- For hypothesis testing the question, the variables that will be included in the model and the allowable  $\alpha$  must be decided first and not changed. For model building, all procedures and cutoffs must be fixed prior to analysis.
- All main effects and any interaction or quadratic effects the investigator suspects are possible should be tested or put into the initial model. Note that

constrained study designs may make testing of some specific interactions impossible.

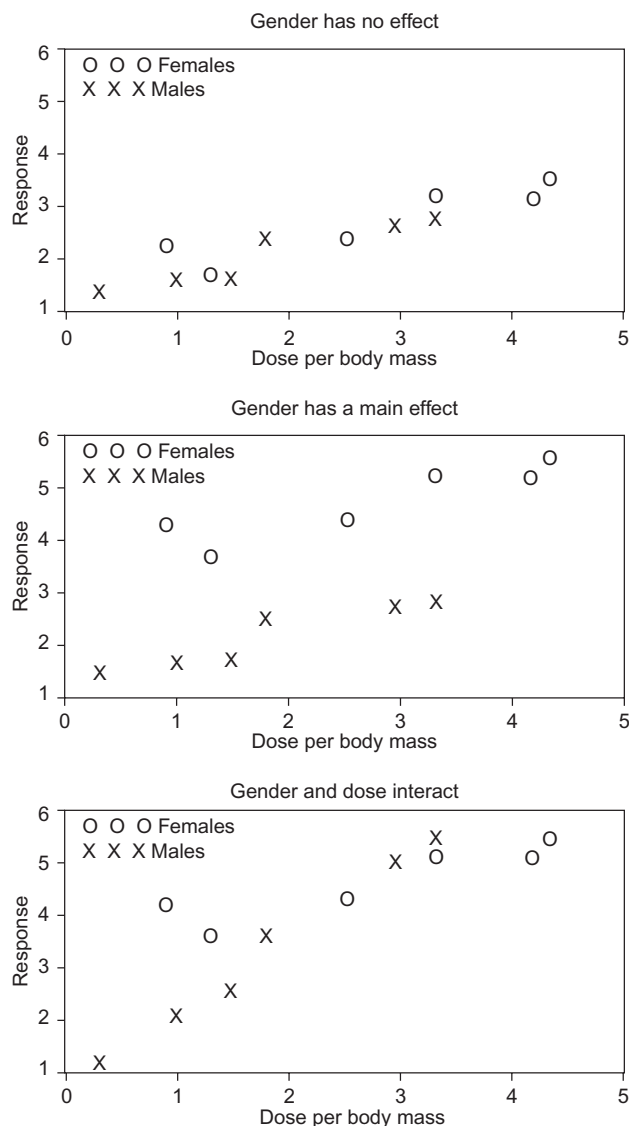
Occasionally, a study does need to be reanalyzed. In that case, the second analysis should be performed by a different person than the first and the second analyst should be told as little as reasonably possible about the first results. Investigators who want further information should look into blinding or, more generally, principles of study design.

## CHOOSING APPROPRIATE TECHNIQUES

### Parametric versus non-parametric tests

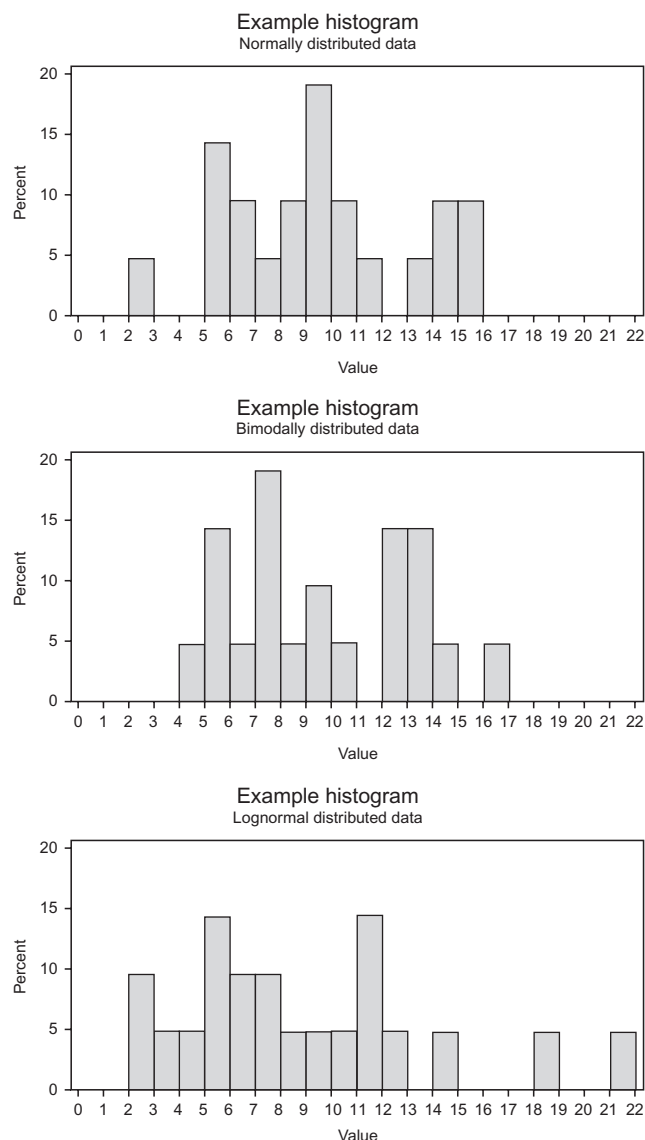
Statistically speaking, the term parametric means the data are assumed to follow a known distribution, usually the normal distribution, while non-parametric means no distribution is assumed. Many statistical tests on dependent interval data have the assumption that the data are normally distributed after adjustment for other model parameters. However, excessive outliers, heteroscedasticity that transformations cannot ameliorate and a few other circumstances can violate this assumption. Moderate departures from normality have little effect on most recommended biostatistics analyses but large deviations can invalidate the results. Therefore, if the investigator finds uncorrectable heteroscedasticity, a larger than expected proportion of outliers, or simply suspects that interval data are non-normal, then they





**FIGURE 12.5** In the first panel, gender has no effect on response, i.e., the dose response (slope and intercept of the best-fit line) is approximately the same for both genders. In the second panel, gender has a main (additive) effect on response. In the third panel, gender and dose interact – the relationship between dose and response depends on gender.

should test to see if the data can be treated as normal for analysis. Graphical analysis can help make this decision but caution is advised. The standard graph for visually examining the distribution of a single variable is the histogram, which shows the data values on the X-axis and the data frequency (count or percentage) on the Y-axis. Figure 12.6 shows three histograms with simulated data from known distributions. Panel one shows data actually drawn from the normal distribution. Unfortunately, normality is not obvious by visual examination. The second panel in Figure 12.6 may look vaguely normal but is actually bimodal.



**FIGURE 12.6** The data in the first panel are random normal with mean 10 and standard deviation 3. The data in the second panel are bimodal, i.e., having two peaks instead of one. Bimodality is common in some blood chemistry readings, e.g., hemoglobin count for males (higher peak) and females (lower peak). Panel three shows data from a lognormal distribution, showing its characteristic asymmetry – more higher values than would be expected from a true normal distribution.

Reasonable tests for normality include Levene's test (Levene, 1960), the Brown-Forsythe test (Brown and Forsythe, 1974) and the Breusch-Pagan, a.k.a. Cook-Weisberg, test (Breusch and Pagan, 1979). Exact choice of test depends on both test availability and the model used. If the test rejects normality then the analysis method should be changed from a parametric method to a nonparametric method (see Table 12.4). Parametric methods are more powerful than nonparametric methods when the data are close to normal but in the cases

TABLE 12.4 Suggested hypothesis testing procedures

Dependent variable(s)	Independent variable(s)				
	Single variable			Multiple variables	
	Interval	Binary	Categorical	Ordinal	Any mixture of interval, binary and/or categorical
<b>Single variable</b>					
Interval, parametric data	ANOVA <sup>a</sup>	Student's <i>t</i> -test <sup>b</sup>	ANOVA	ANOVA <sup>c</sup>	ANOVA
Interval, nonparametric	Nothing recommended	Mann-Whitney U test	Kruskall-Wallace	Kruskall-Wallace <sup>c</sup>	Nothing recommended
Binary	Logistic regression	Fisher's exact test or $2 \times 2$ chi-square <sup>d</sup>	Exact test <sup>d,e</sup> or $R \times C$ chi-square	CMH3 <sup>f</sup>	Logistic regression
Categorical	Nothing recommended	Exact test <sup>d,e</sup> or $R \times C$ chi-square	Exact test <sup>d,e</sup> or $R \times C$ chi-square	CMH3 <sup>f</sup>	Nothing recommended
Ordinal	Nothing recommended	CMH1, CMH2, or CMH3 <sup>f</sup>	CMH1, CMH2, or CMH3 <sup>f</sup>	CMH1, CMH2, or CMH3 <sup>f</sup>	Nothing recommended
<b>Multiple variables</b>					
Interval, parametric data	MANOVA	MANOVA	MANOVA	MANOVA <sup>c</sup>	MANOVA

<sup>a</sup>ANOVA in this case is the same as linear regression, but the statistics will be presented differently.

<sup>b</sup>Special cases: If there are two samples each from a single animal, use a paired *t*-test. If the two groups have significantly different variances, use Cochran's *t*-test.

<sup>c</sup>The best readily available test but not the best overall test – treat the ordinal variable as categorical.

<sup>d</sup>See discussion of Fisher's exact test and chi-square tests to decide which is appropriate.

<sup>e</sup>Not all software packages extend the exact test beyond the  $2 \times 2$  case. If not, combine groups until Fisher's or chi-square can be used.

<sup>f</sup>See separate discussion of Cochran-Mantel-Haenszel statistics.

listed above nonparametric methods are less likely to produce incorrect results.

## Balance

For studies that divide animals into groups or cohorts, even numbers in each group are preferable. This is referred to as balanced group assignment and increases power. However, all of the analysis methods mentioned are valid for moderately unbalanced group numbers.

## Repeated-measures analysis

Body weights and other readings and samples taken from the same animal over time are referred to as repeated measures. Common repeated measures are weight, vital signs, blood chemistry and hematology, and urinalysis values. The preferred techniques for analyzing repeated-measures data is an extension of ANOVA called repeated-measures mixed-model ANOVA, also known as longitudinal analysis. Readings taken close together will be numerically close to one another, especially for slowly changing measures such as animal weights. The term for this is auto-correlation. Therefore, these repeated readings cannot be considered independent samples (or readings), which is a problem because most statistical methods assume sample independence. Repeated-measures ANOVA estimates auto-correlation and thereby improves the estimation of other effects.

## Trend analysis

Trend analysis is a loose grouping of techniques that attempt to determine if there is a consistent relationship between two variables, i.e., as one variable's values increase, the other variable's values consistently increase or decrease (without reversals). Statisticians refer to this type of relationship as monotonic. The simplest monotonic relationship is a linear relationship but shapes such as the logistic curve and Michaelis-Menten curve are also monotonic. Common monotonic trends in veterinary toxicology are body weights of healthy animals, which typically increase over time, treatment response rates, which usually rise with increasing dose, and tumor incidence rates, which usually increase both over time and with increased carcinogen exposure. ANOVA and its many variants are tests for linear trends for interval data and logistic regression is a trend test for proportion of animals responding to treatment based on dose where response is measured as either present or absent. For a 2-by-*k* table, where *k* > 2, the Cochran-Armitage trend test (Cochran, 1954; Armitage, 1955) is recommended. Most of the more advanced trend analyses for toxicologists arise from carcinogenicity studies and three good discussions of this topic are the Food and Drug Administration's guidance on rodent carcinogenicity studies (FDA, 2001), Crowley and Breslow's *Statistical Analysis of Survival Data* (Crowley and Breslow, 1984), and Peto *et al.*'s work for the World Health Organization on carcinogenicity screening (Peto *et al.*, 1980).

## Complex curves

Complex curves such as the Michaelis-Menten curve and the homeostatic response shown in [Figure 12.1](#) can be analyzed using methods that are generally referred to as nonlinear modeling. Pharmacokinetic modeling and estimation of enzyme effects are also naturally nonlinear models. Proficiency with nonlinear models is not common even among biostatisticians but software packages that are designed to correctly analyze pharmacokinetic data include WinNonlin and NLME ([Pharsight, 2010](#)) and acslX ([AEgis, 2010](#)).

## Final choice of hypothesis testing procedure

The exact hypothesis testing method should be selected from [Table 12.4](#) based on variable types and all other considerations mentioned so far. The tests listed are not the only valid tests but are in general robust (resistant to mild departures from assumptions), well accepted and available in virtually all statistical software packages. In more complex cases, consider dividing the data set until it matches one of the cases listed. In cases that are not covered and the situations listed as “Nothing recommended,” consultation with a biostatistician is recommended.

## PERFORMING THE ANALYSIS

The actual methods employed may depend on the chosen software package. Many of the indicated tests can also be performed online, are integrated into software such as Lab Information Management Systems (LIMS) and can even be programmed into a spreadsheet. All of these tests are also available in R ([R Development Core Team, 2008](#)), an open-source statistical analysis package. As a cautionary tale, note that the authors have performed statistical validation on three LIMS and in each case some deviations from validated answers were found. Therefore, if the investigator is using anything other than validated statistical software then the results should be confirmed by two or more sources.

## Hypothesis testing

A hypothesis test chooses between an initial assumption, called the null hypothesis, usually symbolized  $H_0$ , and an alternate assumption, usually  $H_A$  (sometimes  $H_1$ ). In general  $H_0$  is always the hypothesis of no significant relationship while  $H_A$  is the hypothesis of some relationship. The exact form of the relationship depends on the

test run; for example, a test for linear trend could state  $H_0$  as “there is not a linear relationship between  $X$  and  $Y$ ” and the matching  $H_A$  would say something similar to “there is a linear relationship between  $X$  and  $Y$ .” The goal of a hypothesis test is to determine if there is sufficient evidence based on the data to reject  $H_0$ . The pre-determined alpha is the “sufficient” boundary, so if the data fitted to the model are less likely than alpha, then the investigator rejects  $H_0$  and accepts  $H_A$  (generally, that there is a difference). When performing hypothesis testing it is important to understand the assumptions and limitations of the statistical method used. In the example above, a test for linear trend would not necessarily detect more complex relationships.

When selecting a hypothesis, the investigator should decide whether the test should be one-sided or two-sided, a.k.a. one-tailed or two-tailed. One-tailed tests answer questions like “is drug  $Z$  at least as effective as drug  $Q$ ,” “is the tumor incidence higher for exposed animals,” or “is disease progression slower in treated animals.” Two-tailed tests answer questions such as “is blood glucose different for treated animals,” “does treatment  $W$  have equivalent cure rates to treatment  $J$ ,” or “are bacterial mutation rates comparable to controls.” More formally, both a one-sided and a two-sided test have a null hypothesis similar to “ $A = B$ ” but  $H_A$  for the one-sided test will be “ $A > B$ ” or “ $A < B$ ” (one or the other, not both) while  $H_A$  for the two-sided test will be “ $A \neq B$ ” without distinguishing between  $A > B$  and  $A < B$ . When in doubt, use a two-tailed test.

A central consideration in hypothesis testing is control of the chance of false positives. For example, most blood chemistry and hematology panels have 30 or more individual readings. Assuming the investigator tests each reading for statistical significance at  $\alpha = 0.05$ , the actual chance of getting at least one false positive is  $1 - (1 - \alpha)^{30}$ , or about 79.5%. This is called alpha inflation due to multiple comparisons and whether this is acceptable depends entirely on non-statistical factors. For studies on drugs and medical devices, efficacy should be tested with a single measure at  $\alpha = 0.05$ . Both regulatory agencies and professional journals strongly resist accepting studies that performed efficacy tests until the tests showed a positive answer and doing so is statistically invalid. It is reasonable to have secondary efficacy or effect tests, but the study design should specifically state which test is primary and which are secondary. If more than one efficacy test has to be performed, then alpha can be adjusted so that the overall false-positive rate remains at 0.05 or less. The opposite situation applies to analysis of safety information, i.e., in general, all safety parameters are tested at  $\alpha = 0.05$  and all significant results are reported even though this inflates the false-positive rate. One way of understanding this dichotomy is to note drugs or other treatments

frequently have only one intended positive effect so it is important to be certain the positive effect is real but drugs and treatments usually have multiple side effects and it is important to identify all of them. Note that multiple comparisons also inflate the overall false-negative rate but that the false negative rate is rarely controlled.

Reduction of dimensionality may or may not be performed before hypothesis testing – either way is valid as long as the choice is made before analysis. In practice it is seldom done simply because it is an extra unnecessary calculation. When reduction of dimensionality is performed for hypothesis testing it is usually a one-step process where the decision is whether or not covariates, quadratic terms and interaction terms (all terms covered later) should be included in the model or not.

### *Analysis of survival and time-to-event data*

Tests for survival data and time-to-event data are somewhat different than the methods presented in Table 12.4. Common toxicology applications requiring these tests are times to death, tumor occurrence or serious toxicity. For such applications, the dependent variable is the time to the event of interest. Two common tests for survival analysis are the log-rank test (also log rank or logrank) (Mantel, 1966; Peto and Peto, 1972) and the Cox Proportional Hazard test (Cox, 1972). The log-rank test is a simple comparison of two groups while the Cox Proportional Hazard test is capable of handling mixed interval, binary and categorical data as independent variables.

Assumptions and limitations:

- The log-rank test gives the same weight to all calculations over time, even though earlier events are often of more interest. There is a variation called Peto's log-rank test (Peto and Peto, 1972) which weights earlier observations more.
- Hazards between groups in the Cox model are expected to be proportional, i.e., the survival curve for one group is a simple multiple of the survival curve for another group. However, an investigator who suspects non-proportionality based on the survival curve can include a time term in estimation that partially overcomes this limitation.

### *ANOVA and ANCOVA*

ANOVA is essentially the same as linear regression. The statistics are often presented somewhat differently, but the underlying assumptions are identical. Similarly, Analysis of Covariance (ANCOVA) is simply ANOVA with the inclusion of additional independent variables, called covariates, which make the model fit better. For

example, body mass is often a covariate in calculations of drug effects on organ weight. Since organ weights vary by body mass, body mass can explain some of the differences in organ weights, which makes estimation of the remaining differences more accurate. Note that body mass can be a dependent variable and a covariate in the same study, but not in the same analysis.

Interactions between independent variables may be tested by specifying the interaction in the software model or by creating another independent variable that is the multiplication of the values of the variables that are suspected to interact. Similarly, if the software does not allow easy specification of a quadratic model, such models can be tested by creating another independent variable that is the square of the variable that is needed for a quadratic fit. For both quadratic modeling and interaction effects, the original variables must still be included in the model.

For interval independent variables, ANOVA tests for a linear relationship with the dependent variable; for binary independent variables ANOVA tests if the two levels of the binary variable have equal means; and for categorical independent variables ANOVA tests if all groups have equal means. Just knowing means are unequal without knowing which means vary is not normally useful, so other tests to determine differences between levels are used to give a more detailed answer. These secondary tests are called *post hoc* tests, and to preserve the overall false positive rate, they must be used if and only if the first test finds significance. Possible *post hoc* tests for ANOVA are Duncan's (Duncan, 1955), which tests for all possible differences, and Dunnett's (Dunnett, 1955 and 1964), which tests for statistical differences between one specific group and all others and is therefore useful to test for differences from control groups. *Post hoc* tests will sometimes fail to find significance between any paired groups even when the overall test is significant and this lack of result is most common for ordinal data. This situation is not necessarily an error and occurs because results usually have more accuracy based on all the data rather than a subset.

Assumptions and limitations:

- ANOVA assumes the data are normal after adjustment for the independent variables. However, the results of ANOVA are not seriously changed by moderate departures from normality or by modest levels of other analysis limiters such as outliers or heteroscedasticity.
- ANOVA tests for a linear relationship (interval variables) or additive effects (binary or categorical variables). ANOVA can test for quadratic curves but the investigator must specify that before analysis. ANOVA will not correctly test for complex curves.



### Student's *t*-test

Student's *t*-test attempts to determine if the means of the two groups are equal.

Assumptions and limitations:

- The *t*-test assumes the data are normal and homoscedastic (equal variance for all data). The *t*-test is resistant to moderate deviations from assumptions and Cochran's *t*-test can be used for heteroscedastic groups.
- Paired samples, i.e., exactly two samples from the same animal, should use the paired *t*-test.

### Mann-Whitney U test

The Mann-Whitney U test (Mann and Whitney, 1947) is an extension of the Wilcoxon rank-sum test and is also called the Mann-Whitney-Wilcoxon (MWW) test. The MWW test attempts to determine if the two levels of the interval independent variable have equal medians.

Assumptions and limitations:

- The Mann-Whitney test should not be used on paired data. Instead use the Wilcoxon rank sum (Wilcoxon, 1945) for paired data.

### Kruskall-Wallis

The Kruskal-Wallis test (Kruskall and Wallace, 1952) is simply ANOVA on the data replaced by their ranks (1 for the smallest data point, 2 for the next smallest, etc.) and therefore is also applicable to already ranked data. The test can be viewed as testing equality of the medians.

Assumptions and limitations:

- A significant finding does not show which groups are different. To perform *post hoc* tests, examine the groups two at a time with the Mann-Whitney U test.
- Too many tied values will increase the false-positive rate.

### Logistic regression

Strictly speaking, logistic regression is used to predict the probability of an event that can only be measured by its absence (value 0) or presence (value 1), but it can be used to model other dependent binary data also. An example logistic curve is shown in Figure 12.1. The probit model (not shown) is also a reasonable test for similarly shaped responses. Logistic regression should be used instead of Fisher's exact test and chi-square tests when modeling. Common uses include estimating population response rates or toxicity rates based on drug dosage. Like ANOVA, logistic regression can be extended to handle any mixture of interval, binary and categorical

independent variables. The version of logistic regression listed here tests whether the independent variable is correlated with the dependent variable.

Assumptions and limitations (both logistic and probit models):

- The response to independent interval variables is assumed to be monotone, i.e., as the interval variable increases, the likelihood of the response either consistently increases or consistently decreases. Also, both models assume specific shapes and data that do not match those shapes will yield inaccurate or biased results.
- Both models are poor estimators when samples sizes are small.

### Exact tests and chi-square tests

Both chi-square tests and Fisher's exact test (Fisher, 1922) test for independence, i.e., the alternative hypothesis is "some relationship" without defining the relationship. Statistics courses often teach the two-by-two ( $2 \times 2$ ) case as an introduction to the larger, row-by-column ( $R \times C$ ) case but the methods for the  $R \times C$  case encompass the  $2 \times 2$  case. Pearson's chi-square is the computationally simplest and best known chi-square test. Fisher's test was originally described only for a  $2 \times 2$  table, i.e., one independent binary variable and one dependent binary variable, but statisticians have developed similar exact tests for a single categorical independent variable or a mixture of binary and categorical independent variables. However, not all statistical software packages will have exact tests beyond the  $2 \times 2$  case.

Assumptions and limitations:

- Exact tests are too conservative (alpha is not fully controllable and smaller than expected).
- Exact tests are computationally intensive for larger sample sizes.
- Chi-square tests do not show where groups are different. The *post hoc* test for the chi-square is to reduce the data to two groups and test using Fisher's exact test or the  $2 \times 2$  chi-square.
- Chi-square tests require larger data sets to be accurate. To determine whether exact tests or chi-square tests are appropriate, lay out the data in tables with the cell count being the number of observations for each unique combination of the variable values. If any cell is less than 10 in the  $2 \times 2$  case or more than 20% of cells are less than 5 in the  $R \times C$  case, then exact methods should be used, if available. There are some statistical ways around this data volume limitation but the easiest method is for an investigator is to combine categories until either Fisher's or a chi-square test is calculable.

### Cochran-Mantel-Haenszel tests

Cochran, Mantel and Haenszel collaborated on developing statistics that work well for ordinal data (Cochran, 1954; Mantel and Haenszel, 1959; Mantel, 1963), collectively abbreviated CMH statistics. The three tests of note, referred to as CMH1, CMH2 and CMH3 from SAS® terminology (SAS Institute, 2000), ask the following questions:

- CMH1, a.k.a. the Mantel-Haenszel test: Is there a linear association between the dependent and independent variable?
- CMH2: Do the mean scores of the dependent variable vary by levels of the independent variable?
- CMH3: Is there some kind of association between the dependent and independent variables?

Most software packages will have CMH1 and call it the Mantel-Haenszel test. Names and availability vary for the other two tests. Which test is used should be based on the exact question that needs to be answered (linearity, difference in means or association). When in doubt, CMH1 is more readily available and is appropriate for most analysis where the dependent variable is ordinal.

Assumptions and limitations:

- CMH2 and CMH3 do not show where groups are different. The *post hoc* test for the chi-square is to reduce the data to two groups and test using the appropriate test from Table 12.4.
- CMH statistics are approximations and should not be used for data with small cell counts under essentially the same restrictions as the  $R \times C$  chi-square test above.

### MANOVA and MANCOVA

Multivariate Analysis of Variance (MANOVA) and Multivariate Analysis of Covariance (MANCOVA) are extensions of ANOVA for more than one interval dependent variable. MANOVA should be used instead of separate ANOVAs when the investigator suspects interaction between both the independent and dependent variables.

Assumptions and limitations:

- Despite the similarity of names to ANOVA, both the generated statistics and the interpretation of MANOVA are different from ANOVA and beyond the scope of this chapter.

### Model selection

Model selection means production of a statistical model where all of the independent variables in the model have

a strong relationship to the dependent variables. This normally involves dimension reduction because it is rare that all measured independent variables actually have a known direct or strong relationship to the dependent variables. Model selection generally either removes unimportant independent variables from the model (feature selection) or transforms the independent variables to reduce variable count (feature extraction). Most feature selection methods involve three parts:

- A calculated statistic called a metric or scoring metric quantifying how strongly the independent variable(s) are related to the dependent variable(s). The exact statistic used depends on the statistical model used but commonly used statistics are F, Mallows' Cp, Akaike's Information Criterion (AIC) and Bayesian Information Criteria (BIC).
- A cutoff that is compared to the metric to determine when inclusion or exclusion of variables has improved the model. The exact cutoff depends on the statistic used.
- A procedure to decide how independent variables are introduced or eliminated from the models used in intermediate estimations. Examples include forward selection and backward elimination.

A hypothesis testing method quantifies the relationship between variables and therefore can be used in feature selection given an appropriate cutoff and variable selection procedure. However, feature extraction methods usually do not use a hypothesis testing procedure and those methods do not follow the three-part distinction above. The primary limitation of all model selection methods is that the calculated model will fit the specific data used better than it will fit the overall population that the specific data are drawn from. This limitation also implies that the independent variables chosen by model selection are not necessarily those that are important in the whole population. Therefore, in order to make a model usable to the larger population, some form of model validation must be implemented. Model validation involves using data other than the test set that the model was fit for to update the model.

One increasingly common type of model selection problem that is difficult to evaluate is called "high  $p$ , low  $N$ ," indicating the number of potential predictor variables ( $p$ ) is higher than the number of experimental units measured ( $N$ ). Examples in toxicology include attempts to correlate the prevalence or severity of a disease or condition with DNA or mRNA microarray results. Since microarrays can return tens of thousands of results for every sample, there are inevitably more independent variables (predictor variables) than samples. Three good methods of analyzing high  $p$ , low  $N$  data are Principal Component Analysis (PCA), the Least

Absolute Shrinkage and Selection Operator (LASSO or more commonly lasso) and the newer Bayesian Elastic Net. PCA was originally described by Karl Pearson in (Pearson, 1901), although not by that name. The lasso technique was originally described by Robert Tibshirani (Tibshirani, 1996) and is now a family of techniques with specific applications in many areas, including survival modeling. Bayesian Elastic Net (Zou and Hastie, 2005) is relatively new and combines features from several other older techniques. There are also a number of other equally valid techniques for handling high  $p$ , low  $N$  data. Investigators interested in learning more should explore the field of statistical regularization or more generally the fields of data mining and machine learning.

Model selection is in many ways a more complex topic than hypothesis testing, so further expansion is beyond the scope of this chapter.

## INTERPRETING AND DISPLAYING THE RESULTS

The tests listed in Table 12.4 will report some measure of statistical significance for each independent term in the model. Usually the desired answer is the  $p$ -value, which is the probability of the differences seen in the independent variable occurring by chance alone. If the probability is less than the pre-selected  $\alpha$  then the variable is considered significant and the null hypothesis of no relationship is rejected. The way the output presents the  $p$ -value will vary depending on the software and the test but terminology such as " $Pr < F$ ," " $p \leq t$ " and "significance" are some common column headers for  $p$ -values. Many of the tests will also report estimates of effects. Binary, categorical and ordinal effect estimates are reported as differences from one level of the variable's value, e.g., one group is the baseline and the other group estimates are reported as differences from baseline. Linear estimates are the slope of the best fit line. Estimates based on more complex models are best understood by graphing the model's best fit. Software does not always report confidence intervals for the effects but the investigator should request confidence intervals for at least the main variables because they allow more detailed information than a simple accept or reject decision. Confidence intervals are based on  $1 - \alpha$  so with  $\alpha = 0.05$  or 5%, the corresponding confidence interval would be  $1 - 0.05 = 0.95$  or a 95% confidence interval. Interpretation of confidence intervals is tricky – new students of statistics often read a 95% confidence interval as meaning there is a 95% chance that the real value of the effect falls in the interval. However, the real value of the effect is either 100% in the interval or not, so a statement of probability

is misleading at best. A 95% confidence interval really means that if the study were run multiple times in an identical manner then 95% of the resulting confidence intervals would contain the true value of the effect (also assuming the model is correct). For most types of results, confidence intervals that include zero indicate the independent variable has no statistically significant effect. This is true for estimates of risks and odds but for risk and odds *ratios* then confidence intervals that include one indicate no significant effect.

For the relatively simple analyses presented here, any significant effects should be discernible from the graphs or tables and, if not, may indicate a mistake in analysis. Therefore, any significant effects found should be graphed. The estimated effects from some ANOVA models may not be the same as the simple difference of group means. This happens because ANOVA adjusts for other terms in the model and also because ANOVA uses a mean estimation method called least squares mean instead of using the simple mean. The resulting estimates of mean effects can show either a more or less noticeable difference than the original raw means. As an example, examine the first panel of Figure 12.6, the one titled "Gender has No Effect." Simple means of the responses by gender would show some difference in average response. Nevertheless, the slope and intercept of the responses for males and females are essentially identical so ANOVA would report correctly that gender has no effect on response. This is because ANOVA adjusts for the difference in dose per body weight between males and females.

Generally, when interpreting statistical results the investigator should use professional judgment, avoid statements about causation and control the scope of their conclusions based on the various conditions of the test and the analysis. Variables that are highly significant in one study may show as not significant for follow-up studies, so at a minimum the investigator should ask whether significant findings make sense in a larger context and what post-analysis measures could be used to further test the effects found.

## LEARNING MORE

One rapidly expanding field of statistics that was not previously mentioned is Bayesian statistics. Bayesian statistics started with Thomas Bayes (Bayes and Price, 1763), who formalized a method of incorporating prior knowledge into a specific type of probability estimation. The ability to incorporate previous information into analyses is one of the strong points of Bayesian statistics and is important for veterinary toxicologists who

want to use findings from previous studies to improve estimations in future studies. Bayesian statistics are also well integrated into decision theory (quantifying how to make optimal decisions, including resource spending), which is important for investigators who have input into study planning.

Another rapidly expanding field of statistics applicable to toxicology is meta-analysis, which is the study of how to combine results from multiple studies to improve estimates of efficacy or safety. The basic steps are: define the question of interest; determine how the data will be obtained (literature searches are most common); set consistent criteria for which studies are included in the analysis; and select the statistical model which will be used to combine the study information. One weakness of meta-analysis is that it is sensitive to the “garbage in, garbage out” problem, i.e., even a well-executed meta-analysis that uses improperly performed studies can lead to incorrect results. Also, meta-analyses are inherently far more complex than single-study statistical methods, so subtle biases are not readily detectable. Nevertheless, the need for meta-analysis is increasing quickly because of the explosion of available data and careful meta-analyses have significant value. Toxicologists who want a reference that has both introductory and advanced meta-analysis material should consider Sutton, Abrams, Jones, Sheldon and Song’s book *Methods for Meta-Analysis in Medical Research* (Sutton *et al.*, 2000).

For most fundamental biostatistics techniques the internet is the fastest readily available resource, but is often lacking in factual reliability and address stability. Fortunately, most search engines will return formulae, examples and even online tests with searches in the general form: [name of statistical method] [desired result]. Example searches include: Mann-Whitney example, *t*-test online, Pearson’s chi-square formula and Fisher’s exact test limitations. All internet sources should be verified by finding the original reference or from multiple sites. More detail and reliability can be found in online statistical software manuals and biostatistics course notes. Online software manuals usually show up in a general search but can be found by going to the software manufacturer’s site; course notes can be found with either a general search, e.g., “statistics course notes” in a search engine, or a site-specific search of universities with statistic departments. Similarly, many textbooks and references can be searched or read online in part or in full using sites such as Google Scholar (scholar.google.com), Google Books (books.google.com) or book sellers such as Amazon (www.amazon.com). (The authors have no financial or personal interests in these companies.) Ultimately, however, college-level courses followed by practice in the techniques learned are the best way to become proficient in toxicology biostatistics.

If the investigator needs timely, in-depth expertise then the American Statistical Association (ASA) has a searchable list of consultants at <http://www.amstat.org/consultantdirectory/index.cfm> and general web searches for “statistical consulting” or a similar phrase will return a large number of possibilities. Also, university professors and occasionally graduate students perform consulting.

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# Toxicology and the law

Michael J. Murphy

## INTRODUCTION

Much of the law of admissibility of scientific expert testimony arises from toxic tort cases. Consequently, the discipline of toxicology has strongly influenced the legal standards by which scientific expert testimony is admitted in a legal venue.<sup>1</sup>

This chapter uses a hypothetical example of legal issues that may arise in an animal toxicosis case to illustrate application of the law. It is intended to introduce toxicologists, and other scientists, with a broad overview of some of the legal issues that may arise when a medical case becomes a “legal case.” This chapter is not, and is not intended to be, a comprehensive review of the law. Neither is it intended to provide an outline of steps to take for a particular case in a particular jurisdiction.<sup>2</sup>

This chapter primarily relies on the Federal Rules of Evidence, and limited case law, to identify issues<sup>3</sup> that may arise when the findings of a medical diagnosis are used in the courtroom. It focuses on the admissibility of expert testimony in a legal proceeding. However, the reasons for conducting toxicology analyses, jurisdiction, standard of proof and evidence are briefly summarized

to provide a foundation for the role of the expert testimony. The chapter concludes with some questions to consider when analyzing a toxicology case.

## REASONS FOR CONDUCTING TOXICOLOGY ANALYSES

Toxicology has been defined as the scientific *study of adverse effects of chemicals on living organisms* (Eaton and Klaassen, 2001). A toxicologist is one trained to examine the nature of those effects and assess the probability of their occurrence (Eaton and Klaassen, 2001). Exposure, dose response and variability of that response are fundamental principles in the science of toxicology (Eaton and Klaassen, 2001). A showing that the toxicologist offered as an expert witness has applied these fundamental principles to the facts of the case at hand will likely be useful in the determination of whether a toxicologist’s testimony will, or will not, be admissible as evidence. The factors that the judge uses to make this determination are discussed in the expert witness section at the end of this chapter.

A toxicologist relies on analytical toxicology analyses in many toxicology cases. In most instances, it is known at the time the sample is submitted for analytical chemistry analysis that the results of that analysis will be used for legal purposes. Racing chemistry laboratories are one example in the veterinary profession where it is known from the outset that the results of the analytical chemistry testing performed will be used in a legal enforcement setting. In some instances, however, the purpose to which the results will be applied is not known, or,

<sup>1</sup>Venue. The particular county, or geographical area, in which a court with jurisdiction may hear and determine a case. *Black’s Law Dictionary*.

<sup>2</sup>Jurisdiction. It is the power of the court to decide a matter in controversy and presupposes the existence of a duly constituted court with control over the subject matter and the parties.

<sup>3</sup>Issue. A single, certain and material point, deduced by the allegations and pleadings of the parties, which is affirmed on the one side and denied on the other.

more commonly, that purpose changes after the testing is completed.

### **When the reason for testing the sample is known**

Samples are analyzed for the presence of chemical toxins for many reasons and in many settings. Analyses may take place in academic, governmental or private laboratories. Analyses performed in these laboratories may each serve a different purpose, such as research, enforcement actions, preclinical or other regulatory testing, or clinical diagnoses.

Toxicology analyses in academic settings are primarily conducted to support one's research or scholarly efforts. One example is the development of new analytical methods. Another example is the characterization of adverse effects of a particular dose of a particular chemical in a given species. Similarly, researchers may observe the clinical signs and adverse effects of animals exposed to chemicals, then compile these effects in retrospective or prospective case reports in the peer-reviewed literature. Such literature may be useful to support a general causation<sup>4</sup> argument in a legal case. However, the medical case itself, or even the analyses performed on that medical case, may not be admissible in court because of insufficient documentation supporting authenticity. Other laboratories may provide such documentation.

Veterinary diagnostic laboratories normally have some government support to assist in the clinical diagnosis of diseased animals. Such analyses are normally aimed at assisting in the determination of whether an animal may or may not have been exposed to a particular chemical, and, if so, whether the animal has or has not experienced a toxicosis as a result of that exposure. These chemical analyses may give qualitative, semi-quantitative or quantitative results.

Many contemporary analytical chemistry procedures performed in each of the above laboratory types allow detection of the presence of chemicals in biological samples that represent only "background" or "normal" exposure to that chemical. One example is many micro nutrients, such as vitamins A and E, copper, iron, magnesium, selenium and zinc. Similarly, lead may be detected at "background" concentrations in blood, liver or kidney by many analytical toxicology methods available today. Consequently, the detection of the chemical is not "diagnostic" for toxicosis from that chemical, but merely indicates exposure. Information beyond the concentration of a chemical in tissues is often required to reach a toxicosis

diagnosis. The distinction between "exposure to" and "toxicosis from" a chemical is fundamental scientifically and in a legal venue.

Samples are received from many sources. Most diagnostic laboratories accept samples from veterinarians, animal owners, animal industries and others. The circumstances of the collection and handling of the sample prior to submission in the diagnostic laboratory is rarely known with certainty. Similarly, routine protocols in a diagnostic laboratory may be different than those in an enforcement laboratory, particularly with respect to sample tracking, documentation and disposal.

Some government and some private laboratories focus on performing chemical analyses in support of legal enforcement actions. Examples of such laboratories include racing chemistry laboratories, and State or Federal Departments of Agriculture or Health. Many of these laboratories have inspectors who insure proper sample collection and transport to the laboratory. In addition, sample tracking, control, retention and disposal are generally better documented in laboratories devoted to enforcement action than those devoted to research or routine diagnostic testing. The analytical methods used in an enforcement setting have often have been validated by AOACI<sup>5</sup> or a similar entity. This validation often demonstrates that the method has been shown to be reliable in multiple laboratories for quantifying the concentration of an analyte, or a group of analytes, in a specific matrix.

The analytical methods useful in a diagnostic setting may be different from those in an enforcement or research setting. The analytical methods used to support a diagnostic case are often influenced by cost and turn-around time. On the other hand, those in an enforcement setting may be more influenced by reliability of the results reported. These factors directly and indirectly influence the collection of the original sample, tracking of the sample in the laboratory, sample retention, document retention and sample disposal protocols. Protocols appropriate for the development of a method in a research laboratory may not be appropriate for use in an enforcement setting. These factors should be taken into account and samples sent to the appropriate laboratory, when the reason for the analysis is known. The reason is not always known.

### **When the reason for testing the sample is not known or changes after testing**

Problems are more likely to occur if the purpose for which the sample is tested is not known at the times of testing, or changes after the testing is completed. These problems

<sup>4</sup>Causation. The fact of being the cause of something produced or happening. The act by which an effect is produced.

<sup>5</sup>Association of Official Analytical Chemists International.

may give rise to angst on the part of the toxicologist, counsel and others. This angst may occur if sample collection, tracking, analysis and interpretation have not been documented in a way that supports proof of reliability in a legal venue. Questions of authenticity and reliability are more likely to arise in analyses performed in a research, routine clinic or diagnostic laboratory setting than those collected and performed by enforcement personnel. Medical samples submitted and tested for one purpose may not be reliable if used for another purpose. In short, the lack of sample tracking or chain of custody documentation may seriously erode the admissibility of laboratory results. A diagnosis may not be supported without laboratory results and a legal case may not succeed without the diagnosis. Put more bluntly, insufficient sample collection, retention and chain of custody documentation can be a huge problem for a plaintiff or prosecutor in a legal venue. A hypothetical case may help illustrate the application of a number of the following legal concepts.

### Hypothetical case

A hypothetical individual employee of a feed additive manufacturer intentionally adds a foreign chemical to a feed premix during a step in the manufacturing process. The feed premix is distributed to many states. Many local mills purchase the premix and incorporate it into feed in appropriate amounts. The mill then delivers that feed to its customers – animal owners. These animal owners offer that finished feed to their animals in appropriate amounts. These animals ingest the feed, then develop adverse effects. An adverse effect in one of the animals is death. An animal that died after ingesting this feed is submitted to a local diagnostic laboratory by the animal owner. The owner of the animal has not made any association between the new feed and the death of the animal, so the feed is not delivered when the animal is, and the new feed delivery is not part of the history provided to the diagnostic laboratory. The laboratory does a routine necropsy and case workup. A feed additive is mentioned to the animal owner as one of the possible differential diagnostic causes for the lesions seen at necropsy. The owner then takes a grab sample of feed out of the total mixed ration mixer and takes it to the diagnostic laboratory. The chemical is detected in the feed at concentrations sufficiently high to cause the lesions observed in the dead animal.

Various types of legal claims<sup>6</sup> could arise from this case. The results of diagnostic testing may be offered as fact by the animal owner, feed manufacturer, state and

federal agencies, or some combination thereof, against one or more parties. An insurance claim and government enforcement action are discussed very briefly before continuing with issues that arise in civil or criminal litigation.<sup>7</sup>

The results of a routine diagnostic case may now be offered as fact in an insurance claim. The animal owner will claim that the feed provided by the distributor caused the toxicosis experienced by the owner's animals. The animal owner may file a claim with the distributor of the feed for the harm done to her animals. The distributor, or the distributor's insurance company, may settle the claim with the animal owner if all agree. If all do not agree, a civil suit may be filed. The issues that arise in the civil suit are discussed in the next section.

The dispute<sup>8</sup> between the animal owner and the feed distributor involves private parties. The case may also give rise to an enforcement action. These actions may be on the part of one or more agencies in one or more states, the federal government, or any combination of state and federal government. One or more agencies of the state in which the animal was harmed may have statutory authority to protect the health of animals, humans or both. These agencies may include the State Department of Agriculture, the State Department of Health, the State Department of Natural Resources, the State Board of Animal Health or other similar agencies. A state agency may choose to take enforcement action against the feed distributor, premix manufacturer or both in order to protect the health of animals or humans in its state.

The state agency may collect its own samples and perform its own analyses prior to taking any enforcement action. The agency may determine that the concentration of chemical in the feed is greater than a published tolerance or action level for that chemical in that feed. This agency action may end with a fine, recall or other administrative action, if both sides agree.

If both sides do not agree, the feed distributor and premix manufacturer may "appeal" the agency's decision. This appeal may go to an administrative law judge or a similar official within, or outside, the agency, depending on the administrative structure of that particular agency. If the feed manufacturer or premix manufacturer agrees with the ruling of the administrative law judge, the dispute is finished. If all do not agree, the distributor or manufacturer of the feed may choose to appeal the decision of the administrative law judge to a district civil court. This puts the dispute in a venue similar to a civil suit as discussed below.

<sup>7</sup>Litigation. A lawsuit. Legal action, including all proceedings therein. Contest in a court of law for the purpose of enforcing a right or seeking a remedy.

<sup>8</sup>Dispute. A conflict or controversy, a conflict of claims or rights, an assertion of a right, claim, of demand on one side, met by contrary claims or allegations on the other.

<sup>6</sup>Claim. A cause of action. To demand as one's own or as one's right, to assert, to urge, to insist. Means by or through which claimant obtains possession or enjoyment or privilege or thing.



Agencies are given deference at the district court level, making this an increasingly difficult, but not insurmountable, argument to win. The specialty of administrative law is devoted to the details of administrative procedure that arises in such actions. Administrative law is beyond the scope of this chapter.

Federal agencies may also have an interest in the animal case. The Federal Food and Drug Administration (FDA) may have an interest in the case because the feed additive impacted interstate commerce when it crossed state lines, and impacted the health of the public when it caused toxicosis of a food animal. The FDA has authority to protect the public health. It has authority to take administrative action against the manufacturer of the feed similar to that discussed for the state agencies above in that protection of the public's health.

Although agency decisions are normally given deference in the courtroom, regulations developed by agencies may not always address the issue of causation. Agency regulations are often promulgated to protect populations rather than individuals.<sup>9</sup> The affect on an individual referred to as specific causation is discussed in "Specific causation – the differential diagnosis," below. The distinction between enforcement actions taken by agencies to benefit the public as a whole and legally admissible specific causation in an individual animal is not always appreciated.

This distinction may be illustrated by a hypothetical example. A bulk tank load of milk is tested in the receiving bay and found to be positive for the presence of beta-lactam antibiotics using a test kit marketed and validated for this purpose. The entire load of milk is dumped and the producer whose individual sample is positive is charged for the load of milk. Paying for the tanker load of milk in this situation is a contractual agreement between the milk producer and the bulk milk buyer.

The test kit is approved and validated for use in administrative action. That action is to assist in the public policy of preventing exposure of humans to beta-lactam antibiotics to prevent allergic reactions, and to reduce the likelihood of developing strains of bacteria that are pathogenic to humans and resistant to beta-lactam antibiotics. The test kit is sufficient to support the administrative action of preventing these antibiotics from entering the human food supply.

Further analytical chemistry analysis may be required to distinguish penicillic acid from penicillin G from ticarcillin, as an extreme example. This distinction may be important because of the implication raised as to the potential source of the antibiotic in the bulk tank – feed versus a treated cow versus "spiking" the bulk tank. In short, a test that is entirely acceptable for agency action may not be acceptable for a specific type of litigation.

<sup>9</sup>See, e.g., *Troy Corp., AFL-CIO and Simpson*.

This concept is expanded upon in the section on specific causation below. See Dr. Post's chapter on regulatory veterinary medicine for a further discussion of agency issues.

The results of diagnostic testing may rarely be offered as fact in a criminal case. Animal poisoning cases have rarely risen to the level of criminal action. The addition of the chemical to the feed with the intention of causing a toxicosis may be a crime based on animal cruelty or chemical terrorism statutes. The executive branch of government may then choose to prosecute the case. The state – often through its Attorney General's office – may choose to prosecute under an animal cruelty statute. Similarly, the Department of Justice may file criminal prosecution charges if the act is considered to be one of chemical terrorism. In these instances the state or federal government is a direct party in the case.

This discussion of legal venue is not exhaustive. The purpose of this section is merely to indicate how a routine clinical or diagnostic case could become involved in a variety of legal venues including insurance claims, administrative action, civil and criminal litigation at the state and federal levels.

In summary, those samples used for the purpose known at the time of submission to a laboratory can, with some reasonable care, be handled, analyzed, interpreted and stored in a manner consistent with that purpose. Angst may arise on the part of the animal owner, veterinarian, laboratory personnel, counsel and others, in those instances where the application of results of testing are used in a venue not anticipated at the time of sample submission, particularly in those laboratories not accustomed to litigation work. This angst may rise when the documentation of laboratory results is found to not be sufficiently authentic or reliable to support a legal case. The importance of authenticity and reliability are discussed in the evidence section below, immediately after a brief discussion of jurisdiction and standard of "proof."

## JURISDICTION – WHAT ARE THE RULES IN THIS FIGHT AND WHO DECIDES THE WINNER?

The parties have met, discussed and agreed to disagree on reaching a settlement<sup>10</sup> on the insurance claim. They decide to take the case "to court." The owner of the

<sup>10</sup>Settlement. Payment or satisfaction. In legal parlance, implies meeting of the minds or parties to transaction or controversy, an adjustment of differences or accounts, a coming to an agreement.

animals will normally be the plaintiff<sup>11</sup> and the distributor of the feed will normally be the defendant<sup>12</sup> in this legal case. The manufacturer of the premix is also likely to be named as a defendant. The animal owner may be a natural person or may have formed a legal entity<sup>13</sup> like a family farm corporation. The feed manufacturer and premix manufacturer may be businesses organized as sole proprietorships, partnerships, corporations or other legal entities.

A question arises as to “which court” will decide the dispute. The state and federal governments already have authority to rule on disputes between their citizens. This authority ultimately arises from the respective constitutions. State constitutions most commonly delegate authority to the state judicial system to enforce the laws passed by the state legislature. Many state judicial systems have a three tiered structure composed of district courts, courts of appeals and a supreme court, each with increasing authority. Similarly, the federal constitution authorizes the federal judicial system which has district courts, courts of appeals and the U.S. Supreme Court.

Most civil suits that are filed in the United States do not actually go to trial. A settlement is reached in the vast majority of civil cases. This settlement may be reached by the parties on their own, after mediation or arbitration, or even after litigation has begun, but before a judgment is entered.

Mediation and arbitration are two forms of “alternative dispute resolution” (ADR). An outcome reached after ADR may or may not be disputed later in court depending on the final agreement of the parties in the ADR process. Judges often encourage ADR even when civil litigation is filed. Authority may be given by contract. Disputing parties may have agreed not to litigate at all but rather to use ADR. ADR is increasingly used to settle disputes between companies because it can be faster and cheaper than litigation, and because both parties have more control over the outcome of the dispute. Two parties who agree to submit themselves to ADR must grant authority to the mediator or arbitrator at the outset of the deliberation by way of contract. This authority prevails for as long as the contract between the parties is in force. Authority already exists for government.

Parties with an ongoing relationship may have made a contractual agreement to argue any disputes that arise under that contract according to the laws of a particular

state. For example, the manufacturer of the feed additive and the manufacturer of the final feed may have agreed to argue any dispute they may have under that contract according to the laws of Minnesota. One reason for this may be that both parties agree that the cost of arguing jurisdiction for a distributor with outlets in many states, and a manufacturer with plants in many states, is not in the economic best interest of either company.

The parties will need to decide on the state in which the dispute – lawsuit – will be litigated if they have not already made this decision by prior contract. Individuals, and particularly companies, may be citizens of more than one jurisdiction, consequently parties may need to decide which judicial system they choose to “sue out” the case in. This decision may influence the outcome of the dispute because of differences in laws on a particular issue between states. Similarly, federal law on a particular point may be different than that of state law on the same point, so one party may prefer to argue in a federal court and the other in a state court. The choice of venue may then become a significant part of the dispute that must be decided before the merits of the case can be argued. This jurisdictional decision will normally be made by the judge in the venue where the case was originally filed.

Each state has rules governing the procedures to be followed in conducting a civil trial, and procedures to be followed in conducting a criminal trial. Although these procedural rules may have many similarities between states, they are not uniform across all states. The federal rules are more consistent nationwide.

The federal judicial system also has rules governing civil procedure and those governing criminal procedure. These rules are the same across states, although they may be interpreted differently in different federal judicial districts. In short, administrative rules guide administrative actions, state rules guide state civil or state criminal actions and federal rules guide federal civil and criminal actions.

In our hypothetical example, the manufacturer of the premix and the manufacturer of the feed may have agreed to argue any disputes that arise under their business contract according to the laws of Minnesota. The animal owner who chooses to sue the manufacturer of the feed may choose to sue in state court in the state in which the owner fed the feed to their animal, or perhaps in the federal court representing the same geographic area. One argument that may be used to argue that the case belongs in the federal court system is that the feed crossed state lines and therefore impacted interstate commerce. Other elements that may be required to establish federal jurisdiction are beyond the scope of this chapter.

A judgment will be entered by the judge if the dispute goes all the way through trial. Thus the animal owner may receive compensation at the insurance claim,

<sup>11</sup> Plaintiff. A person who brings an action; the party who complains or sues in a civil action and is so named on the record. A person who seeks remedial relief.

<sup>12</sup> Defendant. The person defending or denying; the party against whom relief or recovery is sought in an action or suit to the accused in a criminal case.

<sup>13</sup> Entity. A real being. An organization or being that possesses separate existence for tax purposes.

settlement or judgment level of this dispute if the animal owner prevails.

The point of this section is primarily to indicate that the procedures by which the dispute is handled may be different between states for an insurance claim, arbitration, mediation or litigation. These distinctions between state court systems are beyond the scope of this chapter. On the other hand, the federal judicial system has procedures that are generally applied across the country. They are more similar nationwide, so they are described below to indicate some procedural issues that may arise in toxicology cases. Although many disputes involving animal toxicoses are not tried in federal courts, the federal procedural rules provided below illustrate issues that are likely to be similar across the country.

## STANDARD OF PROOF – HOW SURE DO I NEED TO BE?

One party needs to persuade the other party once a dispute develops. This need is normally referred to as the burden of persuasion in a legal setting.<sup>14,15,16</sup> The burden

### <sup>14</sup>ARTICLE III. PRESUMPTIONS IN CIVIL ACTIONS AND PROCEEDINGS

Rule 301. Presumptions in General in Civil Actions and Proceedings

In all civil actions and proceedings not otherwise provided for by Act of Congress or by these rules, a presumption imposes on the party against whom it is directed the burden of going forward with evidence to rebut or meet the presumption, but does not shift to such party the burden of proof in the sense of the risk of nonpersuasion, which remains throughout the trial upon the party on whom it was originally cast.

<sup>15</sup>Rule 302. Applicability of State Law in Civil Actions and Proceedings

In civil actions and proceedings, the effect of a presumption respecting a fact which is an element of a claim or defense as to which state law supplies the rule of decision is determined in accordance with state law.

<sup>16</sup>Rule 1101. Applicability of Rules

(a) Courts and judges. – These rules apply to the United States district courts, the District Court of Guam, the District Court of the Virgin Islands, the District Court for the Northern Mariana Islands, the United States courts of appeals, the United States Claims Court, and to United States bankruptcy judges and United States magistrate judges, in the actions, cases and proceedings and to the extent hereinafter set forth. The terms “judge” and “court” in these rules include United States bankruptcy judges and United States magistrate judges.

(b) Proceedings generally. – These rules apply generally to civil actions and proceedings, including admiralty and maritime cases, to criminal cases and proceedings, to contempt proceedings except those in which the court may act summarily, and to proceedings and cases under title 11, United States Code.

of persuasion is colloquially referred to as a “burden of proof” – a phrase that toxicologists may find familiar. This burden of persuasion is different in civil cases than it is in criminal cases. Both legal burdens may be different than that of a routine medical diagnosis. The level of medical certainty needed to treat is often presented by quoting the Hippocratic Oath: “first do no harm.” The degree of medical certainty required to treat a disease may only be that needed to give appropriate general and supportive treatment, but not that which would be required to reach an etiological diagnosis. Reaching an etiological diagnosis to any degree of medical certainty is not always done in clinical practice. Similarly, routine cases in a diagnostic laboratory may not reach an etiological diagnosis to any degree of medical certainty either. In many instances reaching an etiological diagnosis is limited by time, cost and the needs of the animal owner and veterinarian. This need may be merely to have guidance for providing supportive treatment. Other limits on the ability to provide an etiological diagnosis may be the available samples, the condition of the samples, the willingness of the owner to spend the money required to reach such a diagnosis and other factors.

This distinction between a medical diagnosis and a “legal” diagnosis is appreciated by the court system. This distinction is summarized in *Wynacht*:

There is a fundamental distinction between Dr. Z...’s ability to render a medical diagnosis based on clinical experience and her ability to render an opinion on causation of W...’s injuries. Beckman apparently does not dispute, and the Court does not question, that Dr. Z is an experienced physician, qualified to diagnose medical conditions and treat patients. The ability to diagnose medical conditions is not remotely the same, however, as the ability to deduce, delineate, and describe, in a scientifically reliable manner, the causes of these medical conditions. *Wynacht v. Beckman Instruments, Inc.* 113 F. Supp. 2d 1205, 1209 (E.D. Tenn. 2000).

In short, the diagnosis reached in a routine medical clinic is not always sufficient to meet a legal burden of persuasion. Similarly, the diagnosis reached in a routine veterinary clinic may not meet this burden either.

The legal burden of persuasion is different in civil and criminal cases. The burden in civil cases is usually either a preponderance of the evidence or clear and convincing evidence. A preponderance of the evidence is also referred to as a “more likely than not” standard. Juries are sometimes instructed that this is a 51% persuasion standard. In a civil case, the plaintiff has the burden of persuading the finder of fact – judge or jury – that his or her claim is valid with a preponderance of the evidence. Similarly, the defense has the burden of persuading the

finder of fact that their defense is valid with a preponderance of the evidence.

The burden of persuasion in criminal cases is much higher. Here, the burden of persuasion is “beyond a reasonable doubt.” The prosecutor has the burden of persuading the fact finder – judge or jury – beyond a reasonable doubt that the crime was committed by the defendant. Conversely, the defendant raises “reasonable doubt” that the prosecution has met this burden. The merits of both civil and criminal cases are argued based on evidence. Some evidence will require interpretation. Interpretation of facts is the role of the expert. The role and types of evidence follow.

## EVIDENCE

Legal cases are decided based on an application of the law to the facts of a particular case. A dispute about facts themselves or interpretation of those facts arises in many legal cases. Disputes of fact are decided by the trier of fact. The trier of fact is the judge in a bench trial. The trier of fact is the jury in a jury trial. The trier of fact learns of these facts largely through evidence.

All facts are not admissible as evidence. The judge must determine which facts are admissible as evidence and which are not as a preliminary matter. Specifically, the judge must determine “the qualifications of a person to be a witness” and “the admissibility of evidence” (Rule 104(a)). The weight and credibility of the evidence is not determined at this preliminary stage. Rather “evidence relevant to weight or credibility” is normally “introduce[d] before the jury” (Rule 104(e)).

The major factor used to determine the admissibility of evidence is relevance. “Relevant evidence” and “material fact” are two phrases that are often used synonymously. “Relevant evidence” is “evidence having any tendency to make the existence of any fact that is of consequence to the determination of the action more probable or less probable than it would be without the evidence” (Rule 401). “[A]ll relevant evidence is admissible” with few exceptions (Rule 401). “Evidence which is not relevant is not admissible” (Rule 402). For example, testimony that the sky is blue may not be admitted as relevant, because the color of the sky has no tendency to indicate that the feed did or did not cause the toxicosis in the animals.

The judge decides which facts are admissible as evidence and which are not. The factors that influence the judge’s decision on admissibility vary with the type of fact. Documents, specimens and testimony are three sources of fact that a party may desire to enter into evidence in toxicology cases.

## Documents

Documents require authentication before they can be considered as evidence. Document authentication is of increasing interest in many laboratories revisiting record retention procedures. Authentication of documents is covered by Rule 901 of the Federal Rules of Evidence.<sup>17</sup> Subsections of this rule specify the procedure for authenticating public<sup>18</sup> or ancient<sup>19</sup> documents. A laboratory will likely have additional influence on its record-keeping. For example, veterinary diagnostic laboratories are adopting ISO standards, contract research laboratories adhere to Good Laboratory Practice standards,<sup>20</sup> many state agencies have state-specific data practices requirements. While the specific requirements may vary, the general purpose is to authenticate the accuracy of the final report.

The case report and the documents supporting that case report may be required to authenticate that report in a legal proceeding. Authentication of case documents is one reason that many laboratories have established record retention procedures. A document and its underlying support are retained for 7 years in some diagnostic laboratories. The specimens themselves are also important in these proceedings.

## Specimens

Specimens are often relevant in toxicology cases. Toxicologists are well aware of the importance of accurate sample identification to establish the relevance of the results of testing a particular sample. Laboratories involved in racing chemistry, regulatory enforcement and GLP studies often have significant sample tracking documents to establish that the result of a test can be tracked to the original specimen. These sample tracking or chain of custody documents are often needed to establish that the sample tested does, in fact, relate to the case report. The absence of sample tracking or such

<sup>17</sup> Rule 901. “The requirement of authentication or identification as a condition precedent to admissibility is satisfied by evidence sufficient to support a finding that the matter in question is what its proponent claims.”

<sup>18</sup> Rule 90(7). “Evidence that a writing authorized by law to be recorded or filed and in fact recorded or filed in a public office, or a purported public record, report, statement, or data compilation, in any form, is from the public office where items of this nature are kept.”

<sup>19</sup> Rule 901(8). “Evidence that a document or data compilation, in any form, (A) is in such condition as to create no suspicion concerning its authenticity, (B) was in a place where it, if authentic, would likely be, and (C) has been in existence 20 years or more at the time it is offered.”

<sup>20</sup> 21 CFR 58.



chain of custody data may eviscerate a legal claim. The federal judicial system is aware of this as well. It considers as relevant evidence "Comparison by the trier of fact or by expert witnesses with specimens which have been authenticated" (Rule 901(3)). Split sample testing in racing chemistry is an example of comparison by an expert witness with authenticated samples. Such split sample testing may be desirable in toxicology cases that reach an insurance claim, civil or criminal case level.

Toxicologists are aware of the potential for different results when different analytical methods are used. This awareness has given rise over the years to recognition of the need to "validate" tests and test results. The federal judicial system is also aware of this need. It requires "[e]vidence describing a process or system used to produce a result and showing that the process or system produces an accurate result" (Rule 901(9)). AOACI is an example of an entity that validates an analytical method for a particular matrix. The plaintiff has the burden of persuading the judge that the analytical results go with the correct sample, and the results of that sample's testing are on the correct report. Authenticating sample results for such proceedings is one reason for standard operating procedures, quality assurance samples, method validation, split sample testing and other such procedures.

Analytical toxicologists know the limits of interpretation of the results of a given test. However, some who see these reports may not be aware of these limits, so toxicologists need to take care in the wording of interpretive comments made.

## Testimony

Testimony is a third means of introducing evidence. Testimony is often gathered during the discovery portion of a legal case by taking the deposition of witnesses. Testimony may also be given orally at trial. Witnesses are under oath both at the deposition and at trial. Testimony given in a deposition may be entered into evidence at trial. This is sometimes done because the witness is not available for the trial date. Testimony may be given in a deposition, and again at trial by the same person on the same subject.

Testimony is given by both "lay" witnesses and expert witnesses. Admission of testimony from a lay witness is evaluated differently than that of an expert witness. A lay witness must have personal knowledge of the facts to which she or he is testifying. Specifically, "[a] witness may not testify to a matter unless evidence is introduced sufficient to support a finding that the witness has personal knowledge of the matter. Evidence to prove personal knowledge may, but need not, consist of the witness' own testimony..." (Rule 602).

This personal knowledge is their recollection of the event. For example, the animal owner in our hypothetical

example may testify that she did purchase the feed, that she fed it to her animals, that she observed certain clinical signs in her animals, and that she delivered a dead animal to the veterinary diagnostic laboratory.

The animal owner may be cross-examined to help verify details of the events. The animal owner's recollection may be refreshed by referring to a written document pursuant to Rule 612.<sup>21</sup>

Examples of a writing to which the animal owner may refer are a receipt and a case report from the diagnostic facility or veterinary clinic. The receipt could be used to refresh the memory of the animal owner as to precisely when and where the feed was purchased. The case report could be used to refresh the memory of the animal owner as to when the animal died, when the diagnostic testing was performed and the results of the testing. An expert witness may be required, however, to interpret the laboratory report.

## ADMISSIBILITY OF EXPERT TESTIMONY

The lay witness is not the only source of testimony. Lay testimony is a source of facts. Some of these facts must be interpreted, which is the role of the expert. Expert testimony is the other main form of introducing testimonial evidence. Expert testimony is commonly used at trial and has become the topic of a number of evidentiary rules. Federal Rules of Evidence 701 through 706 follow in their entirety.<sup>22,23,24,25,26,27</sup>

Expert testimony must not only be relevant as with a lay witness, but it must also be *reliable*. Discussion of this reliability factor constitutes most of the remainder of this

<sup>21</sup> Except as otherwise provided in criminal proceedings by section 3500 of title 18, United States Code, if a witness uses a writing to refresh memory for the purpose of testifying, either –  
(1) while testifying, or

(2) before testifying, if the court in its discretion determines it is necessary in the interests of justice, an adverse party is entitled to have the writing produced at the hearing, to inspect it, to cross-examine the witness thereon, and to introduce in evidence those portions which relate to the testimony of the witness. ...If a writing is not produced or delivered pursuant to order under this rule, the court shall make any order justice requires, except that in criminal cases when the prosecution elects not to comply, the order shall be one striking the testimony or, if the court in its discretion determines that the interests of justice so require, declaring a mistrial. Rule 612. As amended Mar. 2, 1987, eff. Oct. 1, 1987.

<sup>22</sup> Rule 701. Opinion Testimony by Lay Witnesses

If the witness is not testifying as an expert, the witness' testimony in the form of opinions or inferences is limited to those opinions or inferences which are (a) rationally based on the perception of the witness, and (b) helpful to a clear understanding

chapter. In virtually all legal cases, the judge makes preliminary rulings that determine whether the expert may testify at all, and, if so, upon which opinions. Reliability is the primary factor in the judge's determination of admissibility of expert testimony. Two approaches to determine the reliability of expert testimony have evolved, namely the "*Frye* test" and the "*Daubert* test."

## Frye

The *Frye* test is the original standard of reliability for expert testimony, and it remains in several state court systems, including Minnesota. See Table 13.1. The *Frye* test is often summarized as the "general acceptance" standard. A brief history of *Frye* may illustrate its use in a legal setting.

In 1923, a defendant was convicted of second degree murder (*Frye v. United States*). The defendant appealed the trial court's ruling because the court<sup>228</sup> did not allow the defendant to offer expert testimony. The expert testimony would have used a precursor to the "lie detector test" known as the "systolic blood pressure deception test" ("SBPD test"). The defendant's expert claimed that the SBPD test could prove whether or not a person being examined by the SBPD was attempting to deceive the examiner or conceal his guilt with regard to the crime. The judge did not allow this testimony, and the defendant was convicted. The ruling was appealed. The court of appeals held that when "admitting expert testimony deduced from a well-recognized scientific principle or discovery, the thing from which the deduction is made must be sufficiently established to have gained general acceptance in the particular field in which it belongs" (*Frye v.*

of the witness' testimony or the determination of a fact in issue, and (c) not based on scientific, technical, or other specialized knowledge within the scope of Rule 702.(As amended Mar. 2, 1987, eff. Oct. 1, 1987; Apr. 17, 2000, eff. Dec. 1, 2000.)

<sup>23</sup> Rule 702. Testimony by Experts

If scientific, technical, or other specialized knowledge will assist the trier of fact to understand the evidence or to determine a fact in issue, a witness qualified as an expert by knowledge, skill, experience, training, or education, may testify thereto in the form of an opinion or otherwise, if (1) the testimony is based upon sufficient facts or data, (2) the testimony is the product of reliable principles and methods, and (3) the witness has applied the principles and methods reliably to the facts of the case.

Rule 702. (As amended Apr. 17, 2000, eff. Dec. 1, 2000.)

<sup>24</sup> Rule 703. Bases of Opinion Testimony by Experts

The facts or data in the particular case upon which an expert bases an opinion or inference may be those perceived by or made known to the expert at or before the hearing. If of a type reasonably relied upon by experts in the particular field in forming opinions or inferences upon the subject, the facts or data need not be admissible in evidence in order for the opinion or inference to be admitted. Facts or data that are otherwise inadmissible shall not be disclosed to the jury by the proponent of the opinion or inference unless the court determines that their probative value in assisting the jury to evaluate the expert's opinion substantially outweighs their prejudicial effect. Rule 703. (As amended Mar. 2, 1987, eff. Oct. 1, 1987; Apr. 17, 2000, eff. Dec. 1, 2000.)

<sup>25</sup> Rule 704. Opinion on Ultimate Issue

(a) Except as provided in subdivision (b), testimony in the form of an opinion or inference otherwise admissible is not objectionable because it embraces an ultimate issue to be decided by the trier of fact.

(b) No expert witness testifying with respect to the mental state or condition of a defendant in a criminal case may state an opinion or inference as to whether the defendant did or did not have the mental state or condition constituting an element of the crime charged or of a defense thereto. Such ultimate issues are matters for the trier of fact alone. (As amended Oct. 12, 1984.)

<sup>26</sup> Rule 705. Disclosure of Facts or Data Underlying Expert Opinion

The expert may testify in terms of opinion or inference and give reasons therefore without first testifying to the underlying facts or data, unless the court requires otherwise. The expert may in any event be required to disclose the underlying facts or data on cross-examination.

(As amended Mar. 2, 1987, eff. Oct. 1, 1987; Apr. 22, 1993, eff. Dec. 1, 1993.)

<sup>27</sup> Rule 706. Court Appointed Experts

(a) Appointment. – The court may on its own motion or on the motion of any party enter an order to show cause why expert witnesses should not be appointed, and may request the parties to submit nominations. The court may appoint any expert witnesses agreed upon by the parties, and may appoint expert witnesses of its own selection. An expert witness shall not be appointed by the court unless the witness consents to act. A witness so appointed shall be informed of the witness' duties by the court in writing, a copy of which shall be filed with the clerk, or at a conference in which the parties shall have opportunity to participate. A witness so appointed shall advise the parties of the witness' findings, if any; the witness' deposition may be taken by any party; and the witness may be called to testify by the court or any party. The witness shall be subject to cross-examination by each party, including a party calling the witness.

(b) Compensation. – Expert witnesses so appointed are entitled to reasonable compensation in whatever sum the court may allow. The compensation thus fixed is payable from funds which may be provided by law in criminal cases and civil actions and proceedings involving just compensation under the Fifth Amendment. In other civil actions and proceedings the compensation shall be paid by the parties in such proportion and at such time as the court directs, and thereafter charged in like manner as other costs.

(c) Disclosure of appointment. – In the exercise of its discretion, the court may authorize disclosure to the jury of the fact that the court appointed the expert witness.

(d) Parties' experts of own selection. – Nothing in this rule limits the parties in calling expert witnesses of their own selection.

(As amended Mar. 2, 1987, eff. Oct. 1, 1987.)

<sup>28</sup> The judge is referred to as "the court" in legal writing.

TABLE 13.1 The status of *Daubert* in state courts as of 2006<sup>1</sup>

Rejecting <i>Daubert</i>	Adopting or deeming <i>Daubert</i> consistent	Neither rejecting nor adopting
Arizona	Alaska	Alabama
California	Arkansas	Hawaii
Colorado	Connecticut	Illinois
District of Columbia	Delaware	Minnesota
Florida	Georgia	Missouri
Kansas	Idaho	North Carolina
Maryland	Indiana	Virginia
Nevada	Iowa	
New York	Kentucky	
North Dakota	Louisiana	
Pennsylvania	Maine	
South Carolina	Massachusetts	
Washington	Michigan	
Wisconsin	Mississippi	
	Montana	
	Nebraska	
	New Hampshire	
	New Jersey	
	New Mexico	
	Ohio	
	Oklahoma	
	Oregon	
	Rhode Island	
	South Dakota	
	Tennessee	
	Texas	
	Utah	
	Vermont	
	West Virginia	
	Wyoming	

<sup>1</sup>The Status of *Daubert* in State Courts, Martin S. Kaufman, Atlantic Legal Foundation, current to March 31, 2006.

*United States* at 1013). This holding is commonly abbreviated as the “general acceptance” test. The trial court decision was upheld, because the defendant had insufficient proof that the SBPD test had gained “general acceptance.” Consequently, the defendant’s expert was not allowed to testify, so this expert testimony was not entered into evidence, and this evidence was then not available to support an argument to overturn the defendant’s conviction.

*Frye* was a federal criminal case, but the holding was later adopted in federal civil cases. Over time, *Frye* was adopted by most state courts for both civil and criminal proceedings. Minnesota reaffirmed its use of *Frye* in *Goeb v. Tharaldson*. Minnesota Supreme Court cases on the subject are briefly summarized to illustrate differences with the *Daubert* test below.

Minnesota adopted *Frye* in 1952 in the case of *State v. Kolander*. In 1980, Minnesota added an analysis to decide that testimony developed through hypnosis was inadmissible (*State v. Mack* at 768–769). Consequently, the Minnesota test for admissibility of novel scientific evidence has evolved into a two-prong *Frye-Mack* standard. The *Frye* prong first requires that a novel scientific

technique be generally accepted in the relevant scientific community, then the *Mack* prong requires that the particular evidence derived from that test have a foundation that is scientifically reliable (*Goeb* at 814; see also *State v. Anderson*, *State v. Jobe*, *State v. Moore*).

The Minnesota Supreme Court reconsidered the *Frye-Mack* two-prong test after *Daubert* passed the United States Supreme Court.<sup>29</sup> It rejected *Daubert* and sustained *Frye-Mack*, stating:

Having reviewed the cases and the commentary surrounding this issue, we reaffirm our adherence to the *Frye-Mack* standard and reject *Daubert*. Therefore, when novel scientific evidence is offered, the district court must determine whether it is generally accepted in the relevant scientific community. See *Moore*, 458 N.W.2d at 97–98; *Schwartz*, 447 N.W.2d at 424–26. In addition, the particular scientific evidence in each case must be shown to have foundational reliability. See *Moore*, 458 N.W.2d at 98; *Schwartz*, 447 N.W.2d at 426–28. Foundational reliability “requires the ‘proponent of a \* \* \* test [to] establish that the test itself is reliable and that its administration in the particular instance conformed to the procedure necessary to ensure reliability’.” *Moore*, 458 N.W.2d at 98 (alteration in original) (quoting *State v. Dille*, 258 N.W.2d 565, 567 (Minn. 1977).) Finally, as with all testimony by experts, the evidence must satisfy the requirements of Minn. R. Evid. 402 and 702 – be relevant, be given by a witness qualified as an expert, and be helpful to the trier of fact. See *State v. Nystrom*, 596 N.W.2d 256, 259 (Minn. 1999).

The Minnesota Supreme Court has held that “the *Frye-Mack* standard for admission ‘facilitates more objective and uniform rulings’” and reduces the “undesired element of subjectivity \* \* \* [in] evidentiary rulings” (*Schwartz* at 424) that may occur with *Daubert*. The Minnesota approach requires that an appellate court review a novel scientific or non-scientific technique for reliability before an expert may offer it (*State of Minnesota v. DeShay*) including DNA testing by PCR-STR (*State of Minnesota v. Nose*). With very rare exceptions,

<sup>29</sup> There have been no developments in Minnesota since *Mack* and *Schwartz* to convince us that the *Frye-Mack* standard is now incompatible with those same rules of evidence in existence at the time of these decisions. Cf. *Leahy*, 882 P.2d at 328 (reasoning that by applying the *Frye* standard after the adoption of the evidence code, the California Supreme Court had concluded *Frye* was compatible with the code); *State v. Copeland*, 922 P.2d 1304, 1314 (Wash. 1996) (noting that by adopting the rules of evidence and continuing to adhere to *Frye*, the Washington Supreme Court “signaled that *Frye* and the evidence rules coexist as the law of th[e] state [of Washington]”).

a layperson may not testify in the form of opinions or inferences (*Ray v. Miller Meester*).

However, *Frye* is no longer the standard in federal cases. The *Frye* test was superseded in federal courts by the enactment of the Federal Rules of Evidence (*Daubert* at 589). The *Frye* test has also been replaced by the *Daubert* test in many state court systems. See Table 13.1. The remainder of this section is devoted to *Daubert* since it has been adopted by the federal courts and many state court systems to determine whether expert testimony is, or is not, admitted into evidence today.

## Daubert

*Daubert* is one of three United States Supreme Court cases addressing the issue of admissibility of expert testimony. In addition, the federal court system has published a Reference Manual on Scientific Evidence to assist judges in applying these rulings. This Reference Manual contains a section devoted to toxicology and a separate section devoted to medical testimony. Much of the material below is summarized from these sources.

Three cases with rulings on the admissibility of expert testimony have reached the United States Supreme Court in recent years. The cases are *Daubert v. Merrell Dow Pharmaceuticals, Inc.* 509 U.S. 579 (1993), *General Electric Co. v. Joiner*, 522 U.S. 136 (1997), and *Kumho Tire Co. v. Carmichael*, 119 S. Ct. 1167 (1999). Both *Daubert* and *General Electric* were toxic tort cases. *General Electric* and *Kumho* are discussed first.

The holdings in *General Electric* and *Kumho* are of legal review and admission of testimony from non-scientists as experts, so they are summarized briefly. The holding in *General Electric* is primarily of legal interest. In *General Electric*, the Supreme Court held that the correct standard for an appellate court to apply in reviewing a district court's evidentiary ruling is an "abuse of discretion" standard. This holding most commonly has the effect of strengthening the decision made by the district court whether it is to admit or to not admit the expert testimony. Of interest to toxicologists and other scientists is the urging by Justice Breyer in *General Electric* that judges avail themselves of court appointed experts (*General Electric* at ???). *Kumho Tire* is not directly on point for toxicologists either.

The Supreme Court held in *Kumho Tire* that the phrases "technical" and "other specialized" knowledge in the Federal Rule of Evidence 702 allow testimony from those with "skill"- or "experience"-based expertise and not just "science"-based expertise. The Supreme Court held that an expert in tire failure analysis could testify as an expert even though his expertise was "skill" or "experience" based, rather than "science" based (*Kumho* at 1171). The *Frye-Mack* test in Minnesota offers

very few exceptions to the rule that lay witnesses may not give expert testimony. The holdings in *Daubert* are of most direct interest to toxicologists and other scientists.

Expert testimony is different from lay testimony. One difference is the possibility that "junk science" may be used to confuse, rather than assist, the trier of fact. Consequently an additional test of admissibility is applied to expert testimony. Such testimony must not only be relevant, but it must also be *reliable*. This concept is explained in the *Daubert* case. Justice Breyer "believe[s] there is an increasingly important need for law to reflect sound science" (Justice Breyer, in the Reference Manual on Scientific Evidence, p. 5). Justice Breyer also writes in the Manual on Scientific Evidence:

The judge is the evidentiary gatekeeper (*General Electric Co v Joiner*, 522 U.S. 136 (1997); *Daubert v Merrell Dow Pharms Inc*, 509 U.S. 579 (1993))). The judge, without interfering with the jury's role as trier of fact, must determine whether purported scientific evidence is "reliable" and will "assist the trier of fact" thereby keeping from juries testimony that, in Pauli's sense, isn't even good enough to be wrong. (Justice Breyer, in Manual on Scientific Evidence, p. 6)

The purpose of *Daubert's* gatekeeping requirement "is to make certain that an expert whether basing testimony upon professional studies or personal experience, employs in the courtroom the same level of intellectual rigor that characterizes the practice of an expert in the relevant field." (*Kumho Tire Co. v. Carmichael*, 119 S. Ct. 1167 (1999) at 1176. See also *Rosen v. Ciba-Geigy Corp*, 78 F.3d 316 (7th Cir) cert denied, 519 U.S. 819 (1996) at 318)

Clearly, the United States Supreme Court in *Daubert* assigned the trial judge a "gatekeeping" responsibility. This responsibility is to make a "preliminary assessment of whether the reasoning or methodology underlying the testimony is scientifically valid and whether that reasoning or methodology properly can be applied to the facts in issue" (*Daubert* at 589). The "gatekeeper" function requires district court judges to screen proffered expert testimony (*Daubert* at 589). This screening is intended to ensure that expert testimony "is not only relevant, but reliable" (*Daubert* at 589). To be relevant, the testimony "requires a valid scientific connection to the pertinent inquiry as a precondition to admissibility" (*Daubert* at 591-592). Much has been written about the reliability portion of this screening.

To meet the reliability standard, the judge must determine whether testimony is "grounded in the methods and procedures of science" (*Daubert* at 590). Consequently, an expert must account for "how and why" he or she reached the expert opinion (*Kumho* at note 27). The judge's determination is "flexible" (*Daubert*



at 594) so the trial judge has broad latitude to determine *how* to test an expert's reliability (*Kumho* at 1176), and this determination may be different in a criminal case than in a civil one (*Kumho* at 1176). Nevertheless, guidelines for determining the reliability of expert testimony exist.

Four factors were presented in *Daubert* to assist the judge in determining whether a theory or technique has been derived by the scientific method (*Daubert* at 593–594). These four factors are used to determine the theory's testability (*Carmichael* at 1522), because "[w]hether [a theory or technique] can be (and has been) tested" is the "methodology [that] distinguishes science from other fields of human inquiry" (*Daubert* at 593). In short, the four factors are testability, peer review or publication, the existence of known or potential error rates and standards controlling the techniques' operation (*Daubert* at 593).

In summary, the trial judge decides whether or not expert testimony is allowed. The means by which the judge makes this decision is flexible, but the judge often tests both the credentials of the expert and the opinion that expert offers. The expert's opinion must meet "the same standard of intellectual rigor" inside the court room as outside of it (*Kumho* at 1176). This showing of intellectual rigor requires that the expert demonstrate both "how and why" the opinion was reached. This may be done by satisfying four factors, namely (1) whether the theory or technique can be tested, (2) whether it is published or peer reviewed, (3) whether it has known or potential error rates and (4) what the control standards are.

These factors may be applied to both the analytical and interpretive portions of a toxicology case. Both *Daubert* and *General Electric* were toxic tort cases. This may be one reason that a chapter devoted to toxicology appears in the Reference Manual for Scientific Evidence. This Manual offers some questions to be asked of purported toxicology experts.

Three questions may be asked in the evaluation of a toxicology expert to determine whether the expert's testimony is reliable – and therefore admissible. These questions may be reduced to (1) is the expert qualified?, (2) has general causation been established?, and (3) has specific causation been established?

### *Is the Expert Qualified?*

The individual wanting to be allowed to testify as an expert must be "qualified as an expert by knowledge, skill, experience, training, or education, ..." (Rule 702). Toxicologists are often argued to be experts based on education, board certification and other means. Although "...no single academic degree, research specialty, or career path qualifies an individual as an expert in toxicology. ...A number of indicia of expertise can be explored, ..." (Reference Guide on Toxicology, p. 415, old).

The basis of a toxicologist's opinion is often a thorough review of the scientific literature and treatises

disclosing the adverse effects of exposure to a particular chemical (Reference Guide on toxicology, p. 415, new).

### *Has the Person Been Trained and Educated in the Discipline of Toxicology?*

An advanced degree in toxicology presumptively supports the argument that the person has been trained and educated in the discipline of toxicology. One may go to the credentialing and other means portions of the analysis if the person possesses an advanced degree in toxicology. Not all toxicologists possess such a degree, however.

Many well-qualified toxicologists do not have an advanced degree in toxicology, but do have an advanced degree in related disciplines such as pharmacology, biochemistry, environmental health, industrial hygiene or other similar fields (Reference Guide on Toxicology, p. 415). However, not all individuals in possession of these related degrees are toxicologists, so further evidence of training and education is often required of those who do not have an advanced degree in toxicology but do have an advanced degree in a related discipline.

This training and education is often in the form of college level course work or continuing education programs. The number of required courses in toxicology cannot be stated, but one such course is not enough. Specifically, "a single course in toxicology is unlikely to provide sufficient background for developing expertise in the field" (Reference Guide on Toxicology, p. 415). "A physician without particular training or experience in toxicology is unlikely to have sufficient background to evaluate the strengths and weaknesses of toxicological research" (see *Mary Sue Henifin*). "[M]ost physicians have little training in chemical toxicology and lack an understanding of exposure assessment and dose-response relationships" (see *Mary Sue Henifin*). The same is true for veterinarians and many other health care professionals. So, while the number of college courses, continuing education or other training programs required to support an argument that one is a toxicologist is not established, one such course is most likely too few. Consequently, an MD or DVM without an advanced degree in toxicology may well be found to not be a toxicologist based on training and education if they have only a course in professional school to support the claim. Certification is another way to support the argument that one is a toxicologist.

### *Has the Person Been Certified as a Toxicologist?*

Some professions have a subspecialty in toxicology with a board certification. Physicians may be certified by the American Board of Medical Toxicology.<sup>30</sup> Veterinarians

<sup>30</sup>A list of approved medical subspecialties is available at: <http://www.abms.org/approved.asp>

may be certified by the American Board of Veterinary Toxicology.<sup>31</sup> The American Board of Toxicology (ABT) was established to provide certification of individuals trained and experienced in toxicology without consideration of whether the individual has a professional Medical or Veterinary Medical degree.<sup>32</sup> However, individuals with an MD, or DVM, degree may also take the ABT certifying examination. A very few individuals are certified by more than one of these boards. Certification by other boards may be relevant for a particular legal case. A person with an advanced degree in toxicology and board certification has strong support for an argument that they are a toxicologist. This may be one reason that the American Association of Veterinary Laboratory Diagnosticians (AAVLD) requires a board certified toxicologist be on staff for an accredited laboratory. A person with an advanced degree but no board certification, or board certification but no advanced degree, may have other criteria to support a finding that they are a toxicologist.

*Do other Criteria Support an Argument that the Person is a Toxicologist?*

Such other criteria may be membership in one or more toxicology organizations, peer-reviewed or other publications, research grants, scientific advisory panels, university appointments and the like (see *Mary Sue Henifin*, p. 418). These criteria are often documented in one's résumé or curriculum vitae.

So, a person with a professional degree, plus an advanced degree in toxicology, plus board certification in toxicology, plus membership in one or more toxicology organizations, peer reviewed toxicology literature, grants, service on scientific advisory panels, and a university appointment has very strong support for an argument that they are an expert in toxicology. On the other hand, a person with a professional degree, no advanced degree in toxicology, no board certification in toxicology and no other publications, grants and the like has a very weak argument, if any, that they are an expert in toxicology. The courts recognize the distinction between a professional degree and expertise required in a legal setting. This distinction is aptly described in *Wynacht v. Beckman Instruments*:

There is a fundamental distinction between Dr. Ziem's ability to render a medical diagnosis based on clinical experience and her ability to render an opinion on causation of Wynacht's injuries. Beckman apparently does not dispute, and the Court does not question, that Dr. Ziem is an experienced physician, qualified

to diagnose medical conditions and treat patients. The ability to diagnose medical conditions is not remotely the same, however, as the ability to deduce, delineate, and describe, in a scientifically reliable manner, the causes of these medical conditions.

Many combinations of education, certification and other criteria that fall between the two above extremes are possible for toxicology experts. These would, of course, be decided by the judge in a specific case. Once found to be an expert, the toxicologist will most likely be asked to give an opinion on general causation.

*General Causation*

"[T]he methodology prescribed by both the World Health Organization (WHO) and the National Academy of Sciences (NAS) for determining whether a person has been adversely affected by a toxin" have been described in *Mancuso v. Consolidated Edison Co.* as a three-step procedure. Scientific validity may be argued in three steps "(1) the validity of the underlying principle, (2) the validity of the technique applying the principle, and (3) the proper application of the technique on a particular occasion" (*Giannelli*, 1980).

First, the level of exposure of plaintiff to the toxin in question must be determined; second, from a review of the scientific literature, it must be established that the toxin is capable of producing plaintiff's illness – called "general causation" – and the dose/response relationship between the toxin and the illness – that is, the level of exposure which will produce such an illness – must be ascertained; and third, "specific causation" must be established by demonstrating the probability that the toxin caused this particular plaintiff's illness, which involves weighing the possibility of other causes of the illness – a so-called "differential diagnosis." (*Mancuso* at 399)

Toxicologists may arrive at an expert opinion in a variety of ways.

The basis of the toxicologist's expert opinion in a specific case is a thorough review of the research literature and treatises concerning effects of exposure to the chemical at issue. To arrive at an opinion, the expert assesses the strengths and weaknesses of the research studies. The expert also bases an opinion on fundamental concepts of toxicology relevant to understanding the actions of chemicals in biological systems. (*Manual on Scientific Evidence*, p. 415)

Two key legal concepts of causation have emerged as courts have attempted to keep junk science out of the courtroom. These concepts are general causation and

<sup>31</sup> [www.abvt.org](http://www.abvt.org)

<sup>32</sup> [www.abtox.org](http://www.abtox.org)

specific causation. The discussion of specific causation follows in the next section.

"Causation is frequently a crucial issue in toxicology cases. Establishing causation means providing scientific evidence from which an inference of cause and effect may be drawn" (Reference Manual on Scientific Evidence, p. 32). The Manual goes on to describe the process of arriving at general causation.

Once the expert has been qualified, he or she is expected to offer an opinion on whether the plaintiff's disease was caused by exposure to a chemical. To do so, the expert relies on the principles of toxicology to provide a scientifically valid methodology for establishing causation and then applies the methodology to the facts of the case.

An opinion on causation should be premised on three preliminary assessments. First, the expert should analyze whether the disease can be related to chemical exposure by a biologically plausible theory. Second, the expert should examine if the plaintiff was exposed to the chemical in a manner that can lead to absorption into the body. Third, the expert should offer an opinion as to whether the dose to which the plaintiff was exposed is sufficient to cause the disease. (Reference Guide on Toxicology, p. 419)

Courts define general causation as "the capacity of a product to cause injury" (*Siharath* at ???). General causation is a scientifically established cause-and-effect relationship. To satisfy this burden, sufficient testing must be done to establish that a disease or condition can arise after exposure to a certain substance. Peer-reviewed literature of epidemiology studies, case reports, *in vitro* and animal studies may be used to support a general causation argument.

A toxic tort plaintiff must first show that the substance to which he was allegedly exposed is capable of causing his injury – general causation (see, e.g., *Raynor* at 7). General causation asks whether exposure to a substance causes harm to anyone.

Increasingly, the discipline of epidemiology is argued as a basis for general or specific causation. When epidemiology is used to present the incidence and distribution of disease in humans, courts have often ruled the expert opinion admissible. This has not always been the case for toxicological expert opinions because of the need to extrapolate from animal, or *in vitro*, studies to humans. This line of argument may be less persuasive in an animal poisoning case when such extrapolations are not needed.

Occasionally experts rely on state or federal regulations to opine that exposure to a certain amount or concentration of a chemical is associated with a particular adverse event. Merely citing the regulation without

further inquiry as to its derivation may erode the strength of such arguments. It may prove critical to the argument to know whether the regulation was established using a "reasonable certainty of no harm" standard or a "normally renders injurious to health" standard.

The former standard may have included various "uncertainty factors" to extrapolate below measured dose-response data to obtain a safety standard. These uncertainty factors may be explored in a legal venue if one is testifying to a "reasonable degree of medical certainty" that the exposure did in fact cause harm, when the basis for the regulatory standard is a "reasonable certainty of no harm" obtained using "uncertainty factors."<sup>33</sup>

In short, is the alleged chemical capable of causing the disease observed at any dose or exposure? In our hypothetical above, has the chemical that was added to the feed additive been shown to cause the clinical signs and lesions observed in the species of animal in this case? If not, the expert's testimony may not be allowed in the case. If so, an analysis of specific causation would be required.

## SPECIFIC CAUSATION – THE DIFFERENTIAL DIAGNOSIS

General causation answers the question of whether the chemical in question may cause the disease observed. Specific causation is aimed at answering the question of whether the chemical in question did in fact cause the disease in the specific case at hand. This concept has been stated in a variety of ways. Specific causation is "proof that the product in question caused the injury of which the plaintiff complains" (*Siharath*). It is a tendency to show that the person's alleged exposure, in fact, caused his or her condition (*Siharath*; see also D.T. Ralston, 2000). In other words, it is a showing that said exposure was the actual cause of the injury (see, e.g., *Raynor*). An analysis of specific causation answers the question of whether exposure to the specific chemical in question did or did not cause the disease experienced by the plaintiff, on in the hypothetical, plaintiff's animals. The specific causation analysis requires consideration of other potential causes of the disease.

Consideration of other causes of the disease is termed the differential diagnosis. The differential diagnosis is a common occurrence in the practice of medicine. Differential diagnosis evidence is often crucial to show specific causation (see *Lennon*). Without some evidence that the substance in question caused the specific injury

<sup>33</sup>See Ellen K. Silbergeld. The role of toxicology in causation: a scientific perspective. 1 Cts. Health Sci. & L. 374, 378 (1991).

to the specific plaintiff, courts are likely to grant the defendant summary judgment. Courts generally agree that, whenever there are different causes for the plaintiff's disease, an expert must perform a differential diagnosis before testimony will be admitted (see *Gianelli*). Courts accept the general validity of the technique of differential diagnosis (*U.S. v. Downing*). An expert opinion based on a properly performed differential diagnosis analysis is not likely to be inadmissible (*Westberry* at 263). More specifically, "[t]o the extent that a doctor utilizes standard diagnostic techniques in gathering this information, the more likely we are to find that the doctor's methodology is reliable" (*In re Paoli* at 758).

Put differently, "[a]n expert who opines that exposure to a compound caused a person's disease engages in deductive clinical reasoning. ...The opinion is based on an assessment of the individual's exposure, including the amount, the temporal relationship between the exposure and disease and other disease-causing factors. This information is then compared with scientific data on the relationship between exposure and disease. The certainty of the expert's opinion depends on the strength of the research data demonstrating a relationship between exposure and the disease at the dose in question and the absence of other disease-causing factors (also known as confounding factors) (Reference Guide, pp. 422–423; see also *Joseph Sanders*, 1994).

However, simply stating that a differential diagnosis was performed is not enough. This issue was discussed in *Viterbo*:

We do not hold, of course, that admissibility of an expert opinion depends upon the expert disproving or discrediting every possible cause other than the one espoused by him. Here, however, Dr. Johnson has admitted that Viterbo's symptoms could have numerous causes and, without support save Viterbo's oral history, simply picks the cause that is most advantageous to Viterbo's claim. Indeed, Dr. Johnson's testimony is no more than Viterbo's testimony dressed up and sanctified as the opinion of an expert. Without more than credentials and a subjective opinion, an expert's testimony that "it is so" is not admissible. (*Viterbo v. Dow Chemical*)

This formulation is repeated by Judge Becker in *United States v. Downing*:

The process of differential diagnosis is undoubtedly important to the question of "specific causation." If other possible causes of an injury cannot be ruled out, or at least the probability of their contribution to causation minimized, then the "more likely than not" threshold for proving causation may not be met. But, it is also important to recognize that a fundamental

assumption underlying this method is that the final, suspected "cause" remaining after this process of elimination must actually be capable of causing the injury. That is, the expert must "rule in" the suspected cause as well as "rule out" other possible causes. And, of course, expert opinion on this issue of "general causation" must be derived from a scientifically valid methodology. (*Cavallo v. Star Enterprise*)

The order of proving causation is important. General causation should be proven first, then the specific causation differential diagnosis analysis. In virtually all cases, differential diagnosis does not provide general causation – it can only provide specific causation.<sup>34</sup> This may be part of the reasoning that argues that epidemiological data are rarely determinative in a specific causation analysis, and are most commonly used in the general causation argument.

To show that said exposure was the actual cause of the injury, toxic tort plaintiffs must prove the admissibility of their expert testimony in both a general causation and specific causation context by a preponderance of proof (*Daubert* at 593). A successful plaintiff must not only show that, more likely than not, the substance can cause the injury in question, but also that, more likely than not, the plaintiff's specific injury was in fact caused by the substance (*DeLuca*; *Ronald J. Allen*, 1991).

A *Daubert* analysis should be performed. In fact, "a trial court that fails to justify its decision not to use *Daubert* factors risks reversal" (*Black v. Food Lion*). *Daubert* factors may be used when assessing the admissibility of clinical medical testimony (*Moore*). This opinion is because reliable opinions are reached using the "methods and procedures of science." Scientific validity is the foundation of "evidentiary reliability" (*Daubert* at 590; see also *Bert Black et al.*, 1994).

So the expert in our case must not only rule in the chemical added to the feed additive as the cause of the clinical signs and lesions observed, but must also rule out other diseases that cause these clinical signs or lesions in that species of animal.

## APPLICATION OF DAUBERT IN TOXICOLOGY CASES

The application of these legal rules to specific toxicology cases may be useful. Testimony may be weakened if there is no *Daubert* inquiry at all (*Goebel*, 2000).

<sup>34</sup>See, generally, *Raynor v. Merrell Pharmaceuticals*, 101 F.3d 129, 138–139 (D.C. Cir. 1996). See also *Kelly*, *Grimes*, *Rutigliano*, *Hall*, *In re Breast Implant*, *National Bank of Commerce* and *Wynacht*.



The differential diagnosis portion of analyzing specific causation is important. Expert testimony from a forensic toxicologist has been properly excluded for insufficient proof to rule in the chemical in question and to rule out other diseases (*Wills*, 2004). The testimony of a toxicologist was properly excluded because the toxicologist was not a medical doctor and therefore not qualified to offer reliable differential diagnosis analysis (*Plourde*, 2003). On the other hand, testimony was properly allowed from two marine biologists who each performed a differential diagnosis analysis in a case involving marine animals (*Clausen*, 2003).

Dose is important in toxicology cases. Testimony of a treating physician, toxicologist and industrial hygienist were excluded, and the exclusion affirmed because the literature did not support a finding of general causation at the relevant exposure (*Amorgianos*, 2002). Testimony of a toxicologist was properly excluded because he performed no dose assessment and showed no statistically significant link between the chemical and the type of cancer present (*Burleson*, 2004).

Reliance on peer-reviewed literature is also important. A toxicologist's testimony was excluded, but the exclusion was reversed, because the toxicologist's testimony was supported by peer-reviewed literature (*Bocanegra*, 2003).

### SOME QUESTIONS TO CONSIDER WHEN USING LABORATORY RESULTS IN A LEGAL CASE

- 1 Was the legal aspect of the case known at the time the samples were submitted? If not, is the sample tracking or chain of custody sufficiently well documented to establish each relevant step of the analysis? Can the relevance of the documents be authenticated? Does chain-of-custody or sample tracking documentation exist?
- 2 What type of legal venue is the work to be used in? Administrative enforcement, civil litigation, criminal litigation? What standard of persuasion is appropriate for this venue?
- 3 Can the relevance of the analytical results be authenticated? What documentation exists that the samples were collected from the appropriate animal? What documentation exists to demonstrate proper sample identification throughout the laboratory testing process? What documentation exists to demonstrate that the process used produces an accurate result – what validation processes are in place? Is the interpretation of the test results accurate for the type of analytical test performed?
- 4 Is the testimony to be as a lay – or fact – witness?

- 5 Is the testimony to be as an expert witness?
  - A Is the expert a toxicologist?
  - B Does the scientific literature support an argument of general causation?
  - C Was a differential diagnosis performed by the laboratory?
    - 1 Rule-in:
      - a Can the expert provide reliable testimony that the sample received by the laboratory is representative of the diseased animals?
      - b Can the expert provide reliable testimony that the animals were in fact exposed to a toxic dose of the chemical in question? To what degree of certainty does the analytical methodology demonstrate exposure of the animal to the chemical in question?
      - c Can the expert provide reliable testimony that the animals experienced the disease expected from exposure to a toxic dose of the chemical in question? Is reliable testimony of the clinical signs and lesions available?
    - 2 Rule-out:
      - a Can the expert provide a differential diagnosis list?
      - b Can the expert provide reliable testimony that the diseases other than the purported one were ruled out? Can the expert explain how and why these other diseases were ruled out? Were scientifically valid methods used to rule out these diseases?

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# Nervous system toxicity

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## INTRODUCTION

The nervous system is a susceptible target for toxicity due to its complex anatomy, specialized functions, high metabolic requirements, limited ability to repair itself and the potential for life-threatening complications when disequilibrium occurs. A plethora of neurotoxins exists including the following: (1) man-made pesticides and agents of chemical warfare that take advantage of this susceptibility, and (2) all-natural compounds that confer advantages to their organisms such as increased predatory efficiency, avoidance of predation or increased survivability of environmental extremes.

As is the case with any toxicant, exposure to a significant dosage of a neurotoxicant warrants appropriate decontamination in an otherwise healthy and asymptomatic patient (see Chapter 104 of this book). In the symptomatic patient, alleviation of clinical signs may be non-specific or symptomatic (e.g., diazepam for seizures) or specifically antidotal based on the mechanism of toxicity (e.g., pralidoxime for organophosphorus insecticides). Table 14.1 lists examples of antidotes used for select neurotoxins. Clinical signs of nervous system toxicity (Table 14.2) can be divided roughly into stimulatory and depressant categories, although overlap of these categories can occur with varying dosages of a given toxicant and/or class of toxicant. When severe signs are not minimized, potential complications can result including extremes of body temperature and blood pressure, hypoxia/anoxia, disseminated intravascular coagulation, rhabdomyolysis, organ failure and trauma resulting from inability to assess the environment and/or inability to avoid environmental hazards.

The general nature of this chapter precludes discussion of every known neurotoxicant. Instead, neurotoxins are divided into those that affect the structural integrity of the nervous system and those that affect only its functional integrity. Keep in mind, though, that functional integrity of the nervous system can be affected by insults to the structural integrity depending on the severity and location of the insult; also the lack of structural changes in functional toxicoses in no way minimizes the potential for a fatal outcome.

## STRUCTURAL TOXICOSES

Histopathological abnormalities associated with structural toxicoses can be subdivided into neuronopathy, axonopathy or myelinopathy. The following discussion addresses each of these pathologies, providing examples relevant to veterinary toxicology.

### Neuronopathy

A neurotoxicant that results in neuronopathy directly targets the neuronal cell body, resulting in cell death and secondary axonal degeneration. Gliosis, proliferation of astrocytes and/or microglial cells, is a common response to loss of neurons (Anthony *et al.*, 2001). With few exceptions, this type of injury is irreversible. Examples of such toxicants include methyl mercury which preferentially targets the cell bodies of the occipital cortex and the cerebellum via an unproven mechanism. Blindness



TABLE 14.1 Neurotoxicants and associated antidotes (Poppenga, 2004; Roder, 2004b,d; Plumb, 2008)

Neurotoxicant(s)	Antidote(s)
Acetaminophen	Acetylcysteine
Amitraz, brimonidine, xylazine	Atipamezole, yohimbine
Benzodiazepines	Flumazenil
Botulinum toxin	Antitoxin
Coral snake	Antivenom
Ethylene glycol	Fomepizole (4-MP), ethanol
Isoniazid	Pyridoxine/vitamin B <sub>6</sub>
<i>Lactrodectus</i> spp.	Antivenin
Lead	Succimer, calcium EDTA, d-penicillamine
Metronidazole	Diazepam
Pyrethroids	Methocarbamol
Opioids	Naloxone
Organophosphorus insecticides	Pralidoxime (2-PAM), atropine
Scorpion	Antivenom
SSRIs/TCAs	Cyproheptadine
Tetanus toxin	Antitoxin

and motor incoordination are common manifestations of lesions in these areas. In veterinary medicine, methyl mercury intoxication is most likely seen in animals that subsist on a diet of contaminated fish. A classic example involves the cats of mercury-contaminated Minamata Bay, Japan, in the 1950s. Due to their advanced cerebellar ataxia, these cats often fell into the water of the bay and were described as “dancing” or “suicidal” (Smith and Smith, 1975; Francis, 1994). In recent years, awareness of environmental contamination with mercury has created biomonitoring programs which attempt to assess the impact of dietary mercury on piscivorous wildlife such as bald eagles (Hinck *et al.*, 2009).

A second example of a toxicant that can directly target neurons of particular relevance in veterinary medicine is the anthracycline glycoside antineoplastic agent doxorubicin (Adriamycin®). Its potential for bone marrow suppression and cardiac toxicity with veterinary use, however, is much greater than its neurotoxic potential (Anthony *et al.*, 2001; Plumb, 2008). Although doxorubicin exerts its antineoplastic effect via interference with transcription, the neurons of the dorsal root ganglia and autonomic ganglia are most susceptible to injury. The selectivity of the effect is thought to be due to a lack of blood–tissue barrier within the ganglia (Anthony *et al.*, 2001).

Domoic acid, the neuronotoxicant responsible for amnesic shellfish poisoning (ASP) in people and wildlife, is produced by the diatom *Pseudo-nitzschia* spp. The toxin is thought to exert its excitatory and cytotoxic effects on hippocampal cells due to its high affinity for binding to the kainate receptor, which is a glutamate receptor subtype. Cellular excitation ensues, resulting in an influx of Ca<sup>2+</sup> ions, up-regulation of the c-Fos gene and cell death (Jeffery *et al.*, 2004). Domoic acid toxicity has been

TABLE 14.2 Signs associated with neurotoxicoses (Podell, 2000)

Stimulatory	Depressant
Hyperactivity	Obtundation
Vocalization	Stupor
Tremors	Coma
Seizures	Ataxia
Hyperesthesia	Paresis/Paralysis
Hypermetria	Abasia
Other:	
Disorientation	
Paresthesia	
SLUDGE (salivation, lacrimation, increased urination, diarrhea, dyspnea, and emesis)	
Cranial nerve deficits (rare)	

responsible for considerable morbidity and mortality of the California sea lion (*Zalophus californianus*; Gulland *et al.*, 2002).

A fourth example of neuronotoxicosis occurs in large animals with yellowstar thistle (*Centaurea solstitialis*) ingestion by horses. This occurs typically in the dry summer and fall and results in neurologic signs including involuntary lip and tongue movements, difficulty in prehending food, tremors, writhing, possible circling, persistent slow movements (dystonia) and significant weight loss. The disease is also called nigropallidal encephalomalacia as a result of the areas of the brain that are uniquely affected (the globus pallidus and the pars reticularis of the substantia nigra). These regions and the clinical signs draw a striking similarity to Parkinson's disease in people (Burrows and Tyrl, 2001; Sanders *et al.*, 2001) which has been associated with selective loss of dopaminergic neurons (van den Munckhof *et al.*, 2006). Of the guaianolide sesquiterpene lactones isolated from *Centaurea* species, cynaropicrin and an analog of solstitialin are cytotoxic in primary cultures of rat substantia nigra cells, and thus raise suspicion for their role in the toxicosis. Aspartic and glutamic acids, two excitatory amino acid neurotransmitters, are also present in *Centaurea* (Burrows and Tyrl, 2001), but their role, if any, in the mechanism of nigropallidal encephalomalacia has yet to be determined.

A final example of a neuronopathic toxicant is currently a common drug of abuse called methylenedioxymphetamine (MDMA) or “Ecstasy.” MDMA selectively targets serotonergic and dopaminergic cells depending on the species exposed, and long-term, irreversible effects may be seen (Gouzoulis-Mayfrank and Daumann, 2006). Acute physiologic effects in people include tachycardia, hypertension, euphoria, heightened sexual awareness, urinary urgency, nausea, chills, sweating and hyperthermia, among others. Signs consistent with serotonin syndrome (hyperactivity, agitation, mental confusion, hyperthermia, tachycardia and

tremors) have been observed in experimentally exposed rats (Easton and Marsden, 2006). Further research is indicated to define species-specific effects, particularly the extent to which serotonergic or dopaminergic neurons are involved. Veterinary practitioners should be aware of the potential for accidental exposure to small animals.

## Axonopathy

The second class of structural lesions caused by neurotoxicants is axonopathy. With damage to the axon, the neuronal cell body remains intact, but the portion of the axon distal to the lesion degenerates, resulting in a "chemical transection" distal to the lesion that is functionally identical to a physical transection of the axon. This is also known as Wallerian or axonal degeneration. Changes in the Nissl substance, the protein synthetic material comprised of free polyribosomes and rough endoplasmic reticulum, become evident histologically in response to this degeneration. These changes include chromatolysis (dissolution of the Nissl substance) as well as margination of the Nissl and the nucleus to the periphery of the cell body. Not surprisingly, those neurons with axons of greatest length are most susceptible to axonal damage. To highlight the susceptibility of distal axons, a subclassification of axonopathy affecting these axons has been termed "central peripheral distal axonopathy." This is in contrast to "central peripheral proximal axonopathy" which involves axons proximal to the spinal cord (Anthony *et al.*, 2001). A third subclassification of axonopathy has been termed "dying back axonopathy." The latter represents progressive death of the axon toward the cell body with time and continued injury. In the peripheral nervous system (PNS), the prognosis for at least partial regeneration is good, but this is less true in the central nervous system (CNS). Secondary demyelination is also possible with axonal injury (Mandella, 2002). Both sensory and motor axons can be affected.

Many agents cause axonopathies, yet just a few are particularly relevant to veterinary medicine. The first example, commonly used as an antineoplastic drug, is the vinca alkaloid vincristine, which is derived from the periwinkle plant, *Vinca rosea* or *Catharanthus rosea*. Vincristine exerts its therapeutic effect by binding to tubulin, inhibiting microtubular formation, disrupting the formation of the mitotic spindle, and arresting cell division at metaphase (Burrows and Tyrl, 2001; Roder, 2004a). Neurotoxicity in the form of axonal degeneration can occur as a result of disruption of fast axonal transport (rate of 400 mm/day normally) which relies on functional integrity of the microtubules (Anthony *et al.*, 2001). Cats are more sensitive to the neurotoxic potential of vincristine. With discontinuation of therapy and appropriate supportive care, animals exhibiting signs of

peripheral neuropathy may improve over several weeks to months (Roder, 2004a). Colchicine, an antimetabolite derived from the autumn crocus (*Colchicum autumnale*) and the glory lily (*Gloriosa* spp.), also inhibits spindle formation (Burrows and Tyrl, 2001; Roder, 2004a). It is used in veterinary medicine for the treatment of amyloidosis associated with Shar Pei fever (Loeven, 1994) as well as hepatic fibrosis (Plumb, 2008). Although reported rarely in humans, no published reports of peripheral neuropathy in veterinary patients were found.

A more common yet fortunately still rare cause of neurotoxicosis in veterinary medicine is metronidazole. A nitroimidazole antibacterial and antiprotozoal agent used to treat *Giardia* and anaerobic intestinal bacterial overgrowth in small animals (Plumb, 2008), metronidazole can result in a sensory peripheral neuropathy manifesting as proprioceptive deficits (Gupta *et al.*, 2000) as well as CNS effects including ataxia, nystagmus, head tilt and seizure activity (Plumb, 2008). Myelinated fibers are most commonly affected (Anthony *et al.*, 2001). The mechanism is unknown for both peripheral and central effects. Oral and/or IV diazepam has been associated with hastened recovery of metronidazole toxicity in dogs (Evans *et al.*, 2003).

A final example of a class of agents that can cause axonopathy are the organophosphorus compounds (OPs), commonly used as insecticides, that can result in signs of neuropathy 7–10 days post-exposure, termed OP-induced delayed neuropathy or OPIDN. An example of historical significance was the delayed neuropathy associated with the intentional contamination of Jamaican ginger alcohol ("Jake") with tri-*ortho* cresyl phosphate (TOCP), an organophosphorus (OP) compound, during the Prohibition years. The TOCP was present in lindol, a substitute solvent added to the Jake to cut costs. The resulting upper motor neuron spasticity and paralysis were irreversible and affected more than 50,000 people (Woolf, 1995). In addition to humans, hens have also been shown to be very sensitive to OPIDN (Damodaran *et al.*, 2001). The neuropathy associated with OPs is thought to involve slow axonal transport macromolecules such as actin and tubulin, which involves movement of the neuronal cytoskeleton at a rate of 1–4 mm/day (Anthony *et al.*, 2001).

## Myelinopathy

Myelin is produced by the oligodendrocytes of the CNS and Schwann cells of the PNS. It is composed of lipid and forms a sheath around certain axons, namely those of the cranial and spinal nerves. It functions to increase the speed of impulse conduction by creating isolated areas of heightened electrical excitability, termed nodes of Ranvier (Spencer, 2000). Myelin gives white matter

its characteristic appearance. Toxicants that result in myelinopathy may affect the myelin itself or target the cells that produce myelin. The insult may result in loss of myelin (demyelination) or edema of the myelin sheath and subsequent separation of myelin lamellae. Remyelination of segmentally demyelinated areas can occur more so in the PNS than the CNS. When peripheral nerves are remyelinated, the process involves more Schwann cells compared to the initial myelination. As a result, the nodes of Ranvier in remyelinated peripheral nerves are closer (Anthony *et al.*, 2001).

Two examples of toxicants that result in intramyelinic edema and separation of the myelin lamellae are hexachlorophene and bromethalin. The former is an antibacterial agent commonly marketed decades ago as pHisoHex<sup>®</sup> and is still commercially available for the treatment and prevention of *Staphylococcal* infections. The latter is a relatively new rodenticide that has been available for roughly 20 years now and is marketed under a variety of trade names including Assault<sup>®</sup>, Sudden Death<sup>®</sup> and Vengeance<sup>®</sup>. The mechanism by which hexachlorophene and bromethalin cause intramyelinic edema is due to the uncoupling of oxidative phosphorylation (Anthony *et al.*, 2001; Dorman, 2004). This uncoupling results in decreased Na<sup>+</sup>/K<sup>+</sup>-ATPase activity, weakened ion gradients and retention of water in the myelin lamellae (van Lier and Cherry, 1988). The use of mannitol or diuretics early in the course of the disease may reverse mild changes, but continued swelling of the lamellae results in a dramatic increase in intracranial and cerebrospinal fluid (CSF) pressure that is typically unresponsive to therapy. Clinical signs in an acute toxic exposure include muscle tremors, hyperthermia, generalized seizures, hyperexcitability, hyperesthesia and death within several hours of ingestion (4–18 h) for bromethalin. Cats are more sensitive to bromethalin than dogs with a minimum lethal oral dose of 0.45 mg/kg versus 2.5 mg/kg in the dog (Dorman, 2004). At lower dosages, hind limb ataxia and paresis can develop in dogs and cats within 2–7 days of ingestion. Signs may include decreased or absent proprioception, loss of response to deep pain, upper motor neuron bladder paralysis, patellar hyperreflexia and varying degrees of CNS depression. These sublethal effects may be spontaneously reversible with time (1–2 weeks). Histologic lesions consistent with bromethalin and hexachlorophene toxicosis include spongy degeneration (diffuse vacuolation) of the white matter of the CNS. Confirmation of a fatal bromethalin toxicosis can be accomplished by identifying the parent compound and/or its more toxic metabolite, desmethylbromethalin, in the liver. Due to their relative inability to metabolize bromethalin into desmethylbromethalin, guinea pigs are resistant to its toxic effects. Treatment of exposed susceptible species is largely aimed at initial decontamination via induction of emesis

(in those species that can vomit) and administration of multiple doses of activated charcoal prior to the onset of clinical signs (Dorman, 2004).

Another toxicant which results in myelinopathy is inorganic lead. The peripheral neuropathic manifestation of lead intoxication is secondary to the segmental degeneration of myelin in distal motor fibers and is most commonly seen in veterinary medicine with chronic intoxication of horses. Sensory function is spared. Clinical signs may include dysphagia and secondary weight loss, ataxia, dysphonia, laryngeal paralysis (“roaring”) and facial nerve deficits. The CNS and other organs may be affected resulting in seizures, depression, secondary aspiration pneumonia, colic and death (Gwaltney-Brant, 2004c).

## FUNCTIONAL TOXICOSES

Most neurotoxicants exert their functional effects via the exquisitely orchestrated mechanisms involved in neurotransmission yet leave no structural footprint of their activity. This can occur at all levels within the nervous system including the CNS, PNS and autonomic nervous system (ANS). Nervous impulses are chemically mediated across synapses by the release of neurotransmitters from the pre-synaptic terminal. These neurotransmitters then move across the synaptic cleft, bind to their post-synaptic target receptor and effect either an excitatory or inhibitory response in the post-synaptic neuron or muscle (Anthony *et al.*, 2001). Functional neurotoxicants may exert their action by preventing synthesis, storage, release, binding, reuptake or degradation of the neurotransmitter. Interference with axonal transmission via sodium, potassium, chloride or calcium channels and subsequent alteration of action potentials can also result in functional toxicoses (Spencer, 2000; Hansen, 2006). Continual development of new pharmaceuticals targeting these endpoints leads to increased likelihood of toxicity in veterinary patients with accidental exposures, particularly in an overdose situation and/or if species differences in pharmacokinetics exist.

Examples of neurotransmitters include acetylcholine; the catecholamine neurotransmitters dopamine, norepinephrine and epinephrine; the amino acid derivatives serotonin (5-hydroxytryptamine; 5-HT), GABA, glycine, histamine, aspartic acid and glutamic acid; and various neuropeptides including enkephalins, substance P (a neurokinin), orexins, endorphins, vasopressin (antidiuretic hormone, ADH) and thyroid releasing hormone (TRH) (Beasley, 1999; Spencer, 2000). The complex array of neurotransmitters provides many targets for neurotoxicity, both by design and unintentional. A more detailed

discussion follows regarding some of the more common neurotransmitters involved in veterinary neurotoxicoses.

## Acetylcholine

Acetylcholine (ACh) is the neurotransmitter that mediates effects at the neuromuscular junction, at the pre-ganglionic neurons of both the parasympathetic and sympathetic nervous systems of the ANS, and at many of the post-ganglionic neurons of the parasympathetic nervous system. It is the target neurotransmitter of OP and carbamate insecticides, which have been marketed since the 1970s and now are widely available commercially for home and agricultural use (Meerdink, 2004b; Gupta, 2006). Acetylcholinesterase (AChE) is the enzyme that degrades ACh to choline and acetic acid within the synaptic cleft. OP and carbamate insecticides bind to AChE, the former by phosphorylating the enzyme, the latter by carbamylating the enzyme, and prevent its degradative action on ACh. This results in an excess of ACh in the synaptic cleft and continued binding to the post-synaptic receptors. Examples of OPs include disulfoton, malathion, terbufos, phosmet, chlorpyrifos, tetrachlorvinphos and parathion. Examples of carbamates include carbaryl, carbofuran, aldicarb, methomyl and propoxur. The onset of action and severity of signs vary widely among these agents. OPs with a sulfur linkage need to be activated by p450 enzymes in the liver prior to exerting their toxic effects, a process which may take just minutes. Carbamates are active upon absorption but some of them are less likely to cross the blood-brain barrier than the more fat soluble OPs. Cholinergic effects are mediated by both muscarinic and nicotinic receptors. Classic muscarinic effects include excess salivation, lacrimation, increased urination, diarrhea, dyspnea (due to increased bronchial secretions) and emesis (abbreviated as the mnemonic SLUDGE or the alternative DUMBELS which stands for diarrhea, urination, miosis, bronchospasm, emesis, lacrimation and salivation). Nicotinic effects include tremors, weakness and paralysis. CNS effects can range from coma and depression to hyperactivity and seizures (Blodgett, 2006).

The main distinction between the OPs and carbamates is the reversibility of the bond between the insecticide and AChE. OPs phosphorylate the esteratic site of the enzyme. At variable times depending on the type of OP bound, the carbon groups attached to the phosphorus are hydrolyzed and replaced by hydrogen. This process is classically referred to as "aging" and represents the point at which there is no possible functional recovery of that enzyme. The use of pralidoxime (2-PAM) as an antidote to preferentially bind the OP is useful only before the aging process has occurred. In contrast, the affinity of carbamates for AChE is much more labile and

aging does not occur with carbamylation of the enzyme. Therefore, the bond between AChE and carbamates is spontaneously reversible with time ( $t_{1/2}$  = 30–40 min) and precludes the use of 2-PAM. Provided the receptor effects are not life-threatening in the meantime, the prognosis for carbamate toxicoses is generally good. With potent carbamates such as methomyl, however, the signs may be severe enough and the duration of effect long enough for the outcome to be fatal. Competitive inhibition of ACh with intravenous (IV) atropine (0.2–0.5 mg/kg,  $\frac{1}{4}$  of dose IV to effect then remainder intramuscular (IM) or subcutaneous (SC)) is indicated for life-threatening bradycardia and/or dyspnea due to bronchospasm and excess bronchial secretions (Plumb, 2008; Blodgett, 2006).

In addition to the chronic OPIDN (discussed under "Axonopathy," above) and the acute toxicosis with SLUDGE effects, there is an intermediate syndrome that is most commonly seen with more lipophilic OPs and classically with chlorpyrifos exposure in cats. It is thought to arise from a down-regulation of muscarinic receptors with sublethal, prolonged exposures. The clinical signs as a result are predominantly nicotinic in nature because those receptors are not down-regulated. Signs which typically appear within 3–10 days of exposure include generalized weakness (including ventroflexion of the neck due to the cat's lack of a nuchal ligament), anorexia, muscle tremors, seizures, depression and/or death (Blodgett, 2006). Due to the reversibility of the bond of AChE with carbamates, the intermediate syndrome is not typically seen. However, it has been uncommonly reported with aldicarb exposures in people (Waseem *et al.*, 2010) and has also been reported with canine aldicarb exposures (ASPCA APCC, unpublished information, 2010).

Another toxicant with muscarinic effects is the mycotoxin slaframine, produced from the fungus *Rhizoctonia leguminicola*, and primarily associated with clovers (*Trifolium* species). It is a cholinergic agonist which is responsible for the clinical picture of profuse salivation or "slobbers" in affected animals (Meerdink, 2004a). Clinical signs consistent with muscarinic stimulation are also seen with ingestion of mushrooms of the genera *Inocybe* and *Clitocybe*, among others, which contain the toxic principal muscarine (Turner and Szczawinski, 1991). Anatoxin-a<sub>(s)</sub>, a neurotoxin produced by the cyanobacteria *Anabaena* sp., *Aphanizomenon* sp. and *Oscillatoria* sp., inhibits AChE in the PNS. Like slaframine, the toxin does not cross the blood-brain barrier (Roder, 2004c).

Several examples of neurotoxicants exist that antagonize muscarinic effects as well. The classic poisonous plant *Atropa belladonna*, or deadly nightshade, is the source of atropine, a racemic mixture of the tropane alkaloids, D- and L-hyoscyamine (Burrows and Tyrl, 2001). Other belladonna alkaloids include scopolamine aka



hyoscyne. *Datura* sp., commonly known as jimsonweed, thorn apple or devil's trumpet, contains scopolamine and hyoscyamine. Excessive anticholinergic action can result in sinus tachycardia, a dry mouth manifesting as increased thirst, dilated pupils, visual disturbances, ileus, urinary retention, restlessness, muscular twitching, incoordination, delirium (as a result of crossing the blood-brain barrier and entering the CNS), respiratory paralysis and death (Burrows and Tyrl, 2001; Pickrell *et al.*, 2004).

Nicotinic cholinergic receptors alone can be involved with neurotoxicoses. Nature has provided several poisonous plants that stimulate these receptors including *Nicotiana* sp. (varying types of tobacco), *Conium maculatum* or poison hemlock and *Lobelia* or Indian tobacco. The numerous alkaloids present in these plants as well as the cyanobacterial toxin anatoxin-a, most commonly produced by *Anabaena flos-aquae* (Roder, 2004c), exert their neurotoxic effects by initially stimulating nicotinic cholinergic and neuromuscular junction receptors but ultimately resulting in a persistent neuromuscular blocking effect. Signs include muscular weakness, tremors, nausea, vomiting, ataxia, tachypnea, tachycardia, hypertension, mydriasis, staggering, seizures, respiratory failure and death (Panter, 2004a, b, c). Treatment is symptomatic and supportive. Prognosis is good except in cases of large overdoses.

Imidacloprid (Advantage®) is a chloronicotinyl nitroguanide insecticide marketed for flea control in pets and which exerts its effects via possibly both agonist and antagonist actions at the nicotinic ACh receptor. Death of the insect is effected via the same clinical signs as previously described above, yet imidacloprid has a wide margin of safety in mammals due to its poor systemic absorption when applied dermally (as labeled) (Craig *et al.*, 2005) and due to the higher affinity of imidacloprid for insect nicotinic receptors versus mammalian receptors. Acute oral ingestions are usually limited to nausea, salivation and vomiting (Wismer, 2004).

Another mechanism by which ACh can be involved in toxicoses is the lack of release of the neurotransmitter from the pre-synaptic terminal. Botulinum toxin, produced by the anaerobic bacterium *Clostridium botulinum*, exerts its action via this mechanism. Sources of the toxin include ingestion of food contaminated with either preformed toxin or clostridial spores, contamination of a puncture wound with spores and ingestion of spores from the environment. Exposure can be oral, inhaled or absorbed cutaneously through devitalized skin (Bailey, 2006). The toxin consists of a 100kD heavy chain and a 50kD light chain linked by disulfide and non-covalent bonds. Upon ingestion of the toxin, its heavy chain binds synaptotagmin, a pre-synaptic vesicle protein. Once the toxin is internalized within the pre-synaptic terminal, its disulfide bonds are cleaved, releasing the light chain. The free light chain is then available to cleave proteins

that are responsible for the docking and release of ACh vesicles into the synapse.

Seven different botulinum toxins (A–G) have been described. Types C and D are most commonly associated with veterinary species (type C is most common in dogs); types A, B, E and F have caused disease in humans. Although differences exist regarding which proteins are cleaved by each toxin type, the end result is the lack of ACh in the synaptic cleft of the neuromuscular junction (Roder, 2004b). The clinical result is a progressive flaccid paralysis which results in death within 3–10 days. Signs are symmetric and progress from the pelvic limbs toward the thoracic limbs. Cranial nerves may be affected, possibly resulting in megaesophagus, decreased jaw tone, facial paralysis and/or a decreased gag reflex. Autonomic functions may be affected as well (Coleman, 1998). The disease is called limber neck in affected birds. In patients that have been supported with artificial respiration, the duration of effect has been documented to persist for 6–8 months (Kotsonis *et al.*, 2001).

The prognosis for severely affected animals is at best guarded but more realistically poor. Treatment can include debridement of wounds, penicillin therapy and antitoxin (5ml IV or IM) within the first 5 days of exposure. Administration of the antitoxin does not reverse the clinical signs attributed to neurons already affected by the toxin. Due to its equine source and antigenicity, an intradermal test dose is recommended prior to full administration IV or IM. For individuals already severely affected, assisted ventilation is indicated until the patient can breathe spontaneously, yet the duration of therapy required may not be practical in veterinary medicine. Extreme supportive care including assisted or parenteral feeding, physical therapy, manual evacuation of the bladder and intermittent manipulation of body position to prevent hypostatic congestion and formation of decubital ulcers is critical. Some laboratories can confirm the diagnosis with analysis of serum, gut contents and/or feed for the preformed toxin (Roder, 2004b; Bailey, 2006). A mouse inoculation assay and type-specific ELISA testing may also aid in the diagnosis of botulism (Thomas, 1991).

### Catecholamine neurotransmitters

Catecholamines are “fight or flight” neurotransmitters that include norepinephrine, epinephrine and dopamine. Their synthesis begins with conversion of the amino acid L-tyrosine to L-dopa. Removal of a carboxyl group from L-dopa forms dopamine which is acted upon by dopamine  $\beta$ -hydroxylase to form norepinephrine (Spencer, 2000). Norepinephrine is also stored in the adrenal medulla and released into the blood with sympathetic stimulation (Capen, 2001). N-methylation of norepinephrine forms epinephrine, an adrenal hormone which is

only nominally present in the brain. Catecholamines are inactivated by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) enzymes (Beasley, 1999; Spencer, 2000).

In addition to its role at post-ganglionic sympathetic neurons, norepinephrine also mediates effects in the CNS. Adrenergic receptors include  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$  and  $\beta_2$  receptors. Stimulation of  $\alpha_1$  adrenergic receptors, present peripherally, results in vasoconstriction and mydriasis. Stimulation of  $\alpha_2$  adrenergic receptors, present in the CNS, mediates inhibition of norepinephrine, resulting in effects opposite those of  $\alpha_1$  stimulation, namely hypotension. Stimulation of  $\beta_1$  receptors, located predominantly in the heart but also in the kidney, adipose, skeletal muscle and eye, results most notably in an increase in the rate and force of cardiac contraction. The  $\beta_2$  adrenergic receptors (present in skeletal muscles; in the smooth muscle of the bronchi, vasculature and uterus; and also in the liver) mediate vasodilation, bronchodilation, uterine relaxation and enhanced glycogenolysis in the liver (Landsberg and Young, 2001).

Examples of adrenergic toxicosis involving overstimulation of the  $\alpha_2$  receptors include accidental ingestion of brimonidine, the active ingredient in Alphagan® eye drops used in the treatment of glaucoma; ingestion of amitraz-containing Preventic® collars; ingestion of clonidine, a human medication used for hypertension and other indications; and overdosage of the sedative/analgesic xylazine. Exposures involving Alphagan® typically involve puncturing of the bottle with sparing amounts actually ingested. Clinical signs for all four toxicoses include profound hypotension and bradycardia, which can be specifically reversed with either of the  $\alpha_2$  antagonists, yohimbine or atipamezole (Antisedan®). Patients should be closely monitored for recurrence of signs once the effect of the antagonist wears off. The half-life of atipamezole in dogs is longer than yohimbine and may require less frequent administration (Plumb, 2008; Mensching, 2011).

An example of adrenergic toxicosis involving overstimulation of the  $\beta_2$  adrenergic receptors involves the accidental puncture of pressurized albuterol inhalers. Albuterol is a  $\beta_2$  adrenergic agonist used therapeutically to treat the bronchoconstriction associated with asthma. When excessive  $\beta_2$  stimulation occurs, profound hypotension, a reflex tachycardia, and subsequent release of catecholamines can occur. Clinical signs usually include tachycardia with possible ventricular premature contractions (VPCs), tachypnea, hyper- or hypotension (depending on the timeframe relative to exposure and the predominating neurotransmitter effect), behavioral changes including restlessness, agitation, anxiety, largely due to secondary catecholamine release, weakness later in the course, and hypokalemia, sometimes severe, due to an intracellular potassium shift. Treatment is largely supportive with fluid therapy, management of severe tachycardia

with a  $\beta$  blocker such as propranolol, supplementation of potassium as needed and diazepam to address behavioral changes due to secondary norepinephrine release (Mensching and Volmer, 2007).

Sympathomimetics such as amphetamines, cocaine, pseudoephedrine, phenylpropanolamine and ma huang mediate dopaminergic and norepinephrine-induced neurotoxic effects including hypertension, hyperexcitability, tachycardia, tremors, seizures, mydriasis, hyperesthesia, head-bobbing, piloerection and death. Phenothiazine tranquilizers such as acepromazine and chlorpromazine are the mainstay of sympathomimetic overdose therapy due to their post-synaptic blocking effects of dopamine as well as inhibition of its release and increase in its turnover in the CNS. Phenothiazines also block  $\alpha$  adrenergic and cholinergic activity and are antihistaminic (Plumb, 2008; Means, 2004).

## Serotonin

Serotonin (5-hydroxytryptamine) synthesis involves the conversion of the amino acid tryptophan to 5-hydroxytryptophan (5-HTP) followed by the conversion of 5-HTP to 5-hydroxytryptamine. The various physiologic roles of serotonin include regulation of sleep, mood, cognition, appetite and behavior (Spencer, 2000). Due to the popularity of selective serotonin reuptake inhibitors (SSRIs) in human and veterinary medicine and over-the-counter supplements containing 5-HTP for the treatment of depression in humans, accidental overdoses are fairly common in veterinary medicine. Examples of SSRIs include fluoxetine (Prozac®), fluvoxamine (Luvox®), paroxetine (Paxil®) and sertraline (Zoloft®) (Plumb, 2008). The seeds of the West African legume *Griffonia simplicifolia* contain 5-HTP. The clinical picture of serotonin excess, termed serotonin syndrome, includes tremors, seizures, hyperthermia, depression (rarely to the point of coma), disorientation, vocalization, hyperesthesia, ataxia, tachycardia, hypertension, agitation, vomiting and diarrhea. Treatment of the clinical patient is largely symptomatic and supportive, but the serotonin antagonist cyproheptadine specifically mediates the effects of serotonin syndrome. The drug can be given orally or crushed, mixed with water or saline, and administered rectally at a dosage of 1.1 mg/kg in dogs (Gwaltney-Brant, 2004a).

## Glycine

Glycine is an inhibitory neurotransmitter that is synthesized from serine. Glycine acts predominantly in interneurons (Renshaw cells) of the brainstem and spinal cord as well as in spinal sensory, auditory and visual pathways. Two well-known toxicants, tetanus and strychnine, act

by inhibiting glycine's inhibitory effects. Tetanospasmin, a biotoxin produced by the anaerobic bacterium *Clostridium tetani*, is responsible for the prevention of glycine release (Roder, 2004b). The ubiquitous bacterial spores typically enter through a puncture or other anaerobic wound. The lack of glycine inhibition results in unchecked muscle contraction, largely of the powerful extensor muscles of the limbs and the masseter muscles. The stereotypical sawhorse stance and "lockjaw" result within 5–10 days of wound infection. Less severe signs include elevation of the nictitating membrane, which may be the earliest indication of toxicosis in the horse, the most sensitive species (Coleman, 1998). Contracture of facial muscles may result in abnormal wrinkles in the skin, erect ears or an abnormal expression, sometimes referred to as a sardonic grin. Progression of the disease results in an inability to rise and possible seizures. Severe muscular contraction or seizures may be induced by external stimuli. Death is due to an inability of the muscles of respiration to relax and subsequent hypoxia (Roder, 2004b).

Diagnosis of tetanus is based on characteristic clinical signs, history of a wound in which the organism could have proliferated and anaerobic culture of an infected wound. Vaccination with tetanus toxoid is recommended to prevent the disease in horses. Animals with wounds should be treated with penicillin, and the wound should be cleaned routinely to prevent proliferation of the bacteria. A tetanus antitoxin exists but is ineffective for toxin that is already bound and will not reverse existing clinical signs. It can help to prevent progression of the disease, though. The dose for horses and cattle is 10,000–50,000 units SC or IM and 3000–15,000 units for sheep and swine and can be repeated in 7–10 days (Roder, 2004b). Contrary to popular belief, the antitoxin may be extremely cost effective. At the time of this writing, the Colorado Serum Company (Denver, CO) sells 15,000 units for \$22.10 (U.S.) ([www.colorado-serum.com](http://www.colorado-serum.com)). Muscle spasms and seizure activity can be managed by minimization of external stimuli as well as with tranquilizers and muscle relaxants such as acepromazine, diazepam, barbiturates and methocarbamol (Coleman, 1998). The prognosis for severely affected individuals is poor.

Another antagonist of glycine is the neurotoxic bait strychnine, an alkaloid derived from the *Strychnos nux vomica* and *S. ignatii* trees. It binds with high affinity to the glycine receptor and blocks its effects as a result (Patocka, 2009). Extreme muscle rigidity occurs as in the tetanus-intoxicated patient and can rapidly progress to intermittent or continuous seizures within 10–120 min of strychnine ingestion. Anxiety, apprehension, nervousness and tachypnea also may be part of the clinical presentation due to this rapid progression. Death occurs due to hypoxia as a result of contracture of the diaphragm and abdominal and intercostal muscles. The oral LD<sub>50</sub> is

0.5–1.2 mg/kg for dogs; 0.5 mg/kg for horses and cows; 2 mg/kg for cats; and 0.5–1 mg/kg for pigs (Talcott, 2006). In the exposed but asymptomatic patient, aggressive decontamination is warranted. Treatment of the symptomatic patient is limited to symptomatic and supportive care. The agents used to treat muscle spasms, seizures and anxiety in the tetanic patient may also be used in the strychnine-intoxicated patient. The prognosis for symptomatic patients is grave.

## GABA

GABA serves as the predominant inhibitory neurotransmitter in the brain of mammals and is synthesized from glutamic acid. Two main receptor subtypes, GABA<sub>A</sub> and GABA<sub>B</sub>, exist. GABA<sub>A</sub> has at least seven subunits which combine with a chloride channel to form a receptor/ionophore complex. The complex contains binding sites not only for GABA but also for drugs such as the anticonvulsant benzodiazepines and barbiturates. Stimulation of these receptors results in CNS depression, somnolence, fatigue, lethargy, ataxia and muscular incoordination. Paradoxical hyperactivity, excitement or aggression can also occur. In overdoses, CNS and respiratory depression may be extreme leading to hypotension, hypoxia, hypothermia and death (Spencer, 2000; Rudolph *et al.*, 2001).

A discussion of GABA warrants mentioning drugs of the avermectin class, commonly used in veterinary medicine for the prevention of heartworm disease (*Dirofilaria*) in dogs and cats; the treatment of endoparasitism (strongyles, ascarids, bots, threadworms, lungworms, stomach worms and summer sores (*Habronema*, *Draschia* species)) and ectoparasitism (*Sarcoptes*, *Demodex*). Examples of avermectins include ivermectin (Heartgard®), selamectin (Revolution®), milbemycin (Interceptor®), moxidectin and abamectin. Their mechanism of action on target species was thought to be due to the enhanced release of GABA at pre-synaptic neurons, causing parasite paralysis and eventual death (Plumb, 2008). Mealey (2006), however, reports evidence that suggests the target effects to be mediated via glutamate-gated chloride channels in the CNS. Such channels are not present in the mammalian brain, allowing for a wide margin of safety of these drugs in the host animal. Furthermore, in genetically healthy animals, p-glycoprotein, part of the blood–brain barrier, is present in the apical membrane of brain capillary epithelial cells, and serves as an efflux pump to remove avermectins from the brain (Mealey, 2006).

Significant clinical signs can be seen, though, in an acute or chronic avermectin overdose, particularly with a dog that has a p-glycoprotein defect or MDR1 mutation. They include ataxia, CNS depression (potentially to the

point of coma), recumbency, disorientation, mydriasis/apparent blindness, muscle tremors, seizures, respiratory depression, hypothermia, bradycardia, hypoxia and death. The onset of signs is expected to be more rapid and the severity of the clinical picture more extreme in the p-glycoprotein deficient dog. Signs may persist for weeks, but the duration of effect is often difficult to predict based on the severity of signs. Extreme supportive care is indicated for the comatose patient. Assisted ventilation may be necessary. Repeated doses of activated charcoal can be of significant benefit due to the enterohepatic recirculation of avermectin drugs. In recent years, treatment of moxidectin toxicity with an emulsion of intravenous lipids indicates that it may be an effective way to hasten elimination of fat-soluble toxicants (Crandell and Weinberg, 2009). If treated aggressively, with or without intravenous lipids, severely affected dogs can recover without long-term sequelae (Mealey, 2006; Merola *et al.*, 2009).

A unique example of neurotoxicity in the dog relates to a species difference in metabolism. Isoniazid, an antibiotic used in the treatment of tuberculosis, is metabolized by acetylation via the enzyme N-acetyltransferase. Dogs are poor acetylators and, therefore, cannot effectively metabolize the drug. Isoniazid forms a complex with pyridoxine (vitamin B<sub>6</sub>), creating a pyridoxine deficiency. Synthesis of GABA requires the cofactor pyridoxal phosphate. In the absence of sufficient cofactor, the inhibitory neurotransmitter is not made and seizures ensue. Emergency treatment involves IV administration of pyridoxine at a dose equivalent to the mg of isoniazid ingested, if known. Supportive care with fluid therapy is indicated, as may be adjunctive diazepam or other anti-convulsants, particularly if the pyridoxine is not immediately available (Villar *et al.*, 1995).

## Glutamate/aspartate

The primary excitatory neurotransmitters of the brain are the non-essential amino acids glutamate and aspartate. Their synthesis in the brain from glucose and other molecules is tightly regulated. The blood-brain barrier excludes excesses of these excitatory neurotransmitters except in areas of the hypothalamus (arcuate nucleus) and retina where acute cellular degeneration may occur as a result. Three main receptor subtypes exist for glutamate: AMPA (DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid), kainate (KA) and NMDA (N-methyl-D-aspartate) receptors. As previously mentioned (see "Neuronopathy," above), domoic acid is an example of a toxicant that stimulates kainate receptors (Jeffery *et al.*, 2004).

As previously mentioned, glutamate and aspartate may play a role in the pathogenesis of yellowstar

thistle intoxication in horses (Spencer, 2000). The plant is known to contain both excitatory amino acids in addition to several unstable guaianolide sesquiterpene lactones which may degrade to the former to exert their neurotoxic effects (Burrows and Tyrl, 2001).

A limited number of drugs used in veterinary medicine are NMDA antagonists. Ketamine, a dissociative anesthetic used commonly for induction of general anesthesia, blocks the open NMDA ion channel by binding to it. Seizures, due to elevation of CSF pressure, are a potential sequela of ketamine use at therapeutic doses. Amantadine, an antiviral drug that is an NMDA antagonist, is being used in the management of chronic pain. Particularly in the early course of treatment, stimulatory signs in dogs such as agitation may be seen. Seizures, tremors and psychosis have been reported in relatively small overdoses in people (Plumb, 2008).

## Histamine

Histamine is a physiologically active amine that is both a neurotransmitter as well as a mediator of peripheral effects relating to allergic reactions and gastric acid secretion. It is formed from the decarboxylation of L-histidine. Peripherally, histamine receptors are of two subtypes: H<sub>1</sub> and H<sub>2</sub>. The former mediate allergic reactions in which histamine is released from the granules of mast cells, resulting in dilation and increased permeability of capillaries (wheal and flare reaction) and constriction of bronchial smooth muscle. The H<sub>2</sub> receptor stimulation increases gastric acid secretion (Gwaltney-Brant, 2004b).

Antagonists of H<sub>1</sub> receptors are commonly used to treat acute and chronic allergic reactions, to prevent mast cell degranulation during surgical excision, as well as to minimize motion sickness in veterinary and human medicine. They are divided into first- and second-generation antagonists. The first-generation antihistamines can both stimulate and depress the CNS while the second-generation antihistamines are considered non-sedating due to their exclusion from the brain at therapeutic dosages. At therapeutic doses and with mild overdoses, particularly with the first-generation antihistamines, CNS depression and hypotension may be seen. Epinephrine is contraindicated for management of hypotension in these patients. Paradoxical CNS excitation including restlessness, hyperactivity, tachycardia, tremors and seizures may also occur and the potential for it varies largely with the individual. Large overdoses of either antihistamine class can result in extreme CNS and cardiovascular (CV) stimulation to the point of seizure activity, cardiac arrhythmias, hypertension and death. Seizures, tremors and hyperactivity may be treated with diazepam or barbiturates (Gwaltney-Brant, 2004b).



Common H<sub>2</sub> blockers used in veterinary medicine to decrease gastric acidity include cimetidine, ranitidine and famotidine. Of the H<sub>2</sub> blockers, cimetidine is more likely to cross the blood–brain barrier. Adverse effects are rare in veterinary patients (Plumb, 2008).

### Mixed neurotransmitter effects

Many pharmaceuticals and toxicants exert their effects through multiple neurotransmitters. One example group is the many ergot alkaloids produced by fungi of the *Claviceps* genus. The mycotoxins can be further divided into ergopeptine and ergoline alkaloids. The alkaloids exert their toxic effects via effects on dopaminergic, serotonergic and adrenergic pathways. Successful recovery is aimed at early diagnosis, when signs such as ataxia and confusion are reversible, and removal of the contaminated feed source (Evans *et al.*, 2004). The effects of the ergoline alkaloids, in particular, can also be seen with ingestion of the seeds of *Ipomoea* or morning glory and are very similar to those of the related street drug lysergic acid diethylamide, also known as LSD (Burrows and Tyrl, 2001).

$\alpha$ -Latrotoxin, the toxin in the venom of the black widow spider (*Latrodectus*), causes the release of catecholamine neurotransmitters as well as ACh, GABA and glutamate. The toxin mediates its catecholamine effects by forming a transmembrane pore through which Ca<sup>2+</sup> can influx, allowing release of pre-synaptic vesicles containing neurotransmitters. Clinical signs associated with a bite from *Latrodectus* species include abdominal pain, ataxia, muscle fasciculations, muscle rigidity and flaccid paralysis, which can ascend to involve the respiratory muscles. Cats are more sensitive than dogs to the effects of  $\alpha$ -latrotoxin, but all mammalian species are susceptible. Antivenin is commercially available for black widow spider bite victims. Like all products derived from equine serum, a test dose should be administered intradermally to determine if hypersensitivity to the product exists. If none exists, the contents of the antivenin vial (2.5 ml) can be diluted 4–20  $\times$  (10–50 ml) with sterile saline and administered slowly intravenously over approximately 15 min. A slow, IV injection of 10% calcium gluconate may be given to treat the muscle fasciculations and weakness as well. Aggressive pain management with opioids or benzodiazepines is indicated (Roder, 2004d).

## ION CHANNELS

The propagation of an impulse along an axon depends on an electrochemical gradient that is intricately regu-

lated by various ion channels in an excitable membrane. Initiation of action potentials, release of neurotransmitters, axonal transport and healthy muscle activity rely on control of ions such as sodium, potassium, chloride and calcium (Spencer, 2000). Specific veterinary examples follow regarding the variety of natural and synthetic toxicants that target the ion channels of the nervous system.

### Sodium channels

The sodium channel of mammals is made up of three protein subunits, the largest of which is the transmembrane  $\alpha$  ( $\alpha$ ) subunit which contains the Na<sup>+</sup> pore and which is flanked by two smaller  $\beta$  subunits. The pores are voltage-gated, allowing sodium ion entrance into the cytoplasm only when charge-dependent conformational changes occur. Nature provides us with several examples of neurotoxicants which affect the sodium channel. Tetrodotoxin (TTX), present in various genera of puffer fish, in the venom of the blue-ringed octopus (*Hapalochlaena* spp.), the rough-skinned newt (*Taricha* spp.) and many other creatures, exhibits a profound binding affinity for the sodium channel peptide complex. Whereas the Na<sup>+</sup> ion normally reversibly binds to the peptide complex for just nanoseconds, TTX binds to the external surface of the peptide ion channel, remains bound for tens of seconds and halts the action potential. A progression of signs occurs with TTX poisoning in people: (1) initial numbness around the mouth with or without nausea; (2) numbness of the tongue, face and skin, early motor paralysis and incoordination manifested as slurred speech; (3) more widespread paralysis resulting in dyspnea, hypotension, an inability to speak and fixed and dilated pupils; and (4) severe paralysis involving respiratory muscles, hypoxia, hypotension and cardiac arrhythmias. Consciousness may be lost followed by death due to respiratory failure as early as 17 minutes after ingestion or as late as 24 hours. No antidote currently exists. Treatment is aimed at decontamination and symptomatic and supportive care (Kaku and Meier, 1995; White, 1995).

Saxitoxin, the causative toxin for paralytic shellfish poisoning (PSP), is produced by several dinoflagellates, most notably *Alexandrium* and *Pyrodinium* species, and causes a similar clinical picture as that of TTX toxicosis also due to binding to the sodium ion channel (Smart, 1995).

Local anesthetics exert their pharmacologic effects by binding to the fast sodium channel complex when it is inactive, inhibiting its recovery after depolarization. Lidocaine, as an example, rapidly dissociates from the sodium channel complex and is also rapidly metabolized in the liver. Consequently, adverse events associated with therapeutic dosages are rare. In an overdose

situation, however, CNS depression, ataxia, seizures and circulatory collapse can occur (Welch, 2000; Plumb, 2008). Dibucaine HCl is 10 times more potent than lidocaine and much more likely to result in adverse effects as a result. Cats are especially susceptible to the methemoglobinemic potential of local anesthetics. Treatment of clinical signs is largely symptomatic and supportive, aimed at maintenance of blood pressure, addressing cardiac arrhythmias and management of seizures (Welch, 2000). The experimental report of bupivacaine-intoxicated dogs successfully treated with intravenous lipid emulsion (Weinberg *et al.*, 2003) holds promise for future use with local anesthetic toxicoses.

Several toxins affect the sodium ion channel by enhancing its activity, resulting in repetitive and prolonged neurotransmission. Brevetoxins, produced by the dinoflagellates *Karenia brevis* and *Gymnodinium breve*, prolong the opening of the sodium ion channels, perpetuating the propagation of the action potential. Bossart *et al.* (1998) implicated the toxin in the death of a minimum of 149 manatees along the southwest coast of Florida in 1996. Another example are ciguatoxins which are produced by dinoflagellates and are bioaccumulated by herbivorous, and then piscivorous, fish. They enhance  $\text{Na}^+$  permeability through the ion channel (Spencer, 2000). The hallmark clinical sign of ciguatera fish poisoning (CFP) in people, and presumably in affected veterinary species, is a paresthesia (tingling sensation) in the extremities as well as a perception of heat with cold stimuli ("dry-ice phenomenon") (Kaplan, 2000). The neurologic signs are attributable to edema of the adaxonal Schwann cell cytoplasm and can persist for weeks. Gastrointestinal pain and diarrhea are also typical, particularly in the early course of the disease (Glaziou *et al.*, 1995; Spencer, 2000).

Other examples of toxicants that block inactivation of sodium channels include batrachotoxin, a steroid alkaloid isolated from the skin of *Phylllobates aurotaenia* and other species of South American poison dart frogs (Spencer, 2000); grayanotoxins, diterpene alkaloids from the plants of the *Rhododendron*, *Kalmia*, *Pieris*, *Leucothoe* and *Lyonia* genera (Puschner, 2004); aconitine, present in monkshood (*Aconitum* spp.); and veratridine from *Veratrum album* (Spencer, 2000). Both  $\alpha$ -scorpion and  $\beta$ -scorpion toxins bind to  $\text{Na}^+$  channels, albeit different sites, and enhance nerve conduction. Toxins of the Sydney funnel web spider (*Atrax robustus* and *Hadronyche* species;  $\delta$ -atraxotoxin, atraxotoxin, robustoxin) also block sodium channel inactivation. Persistent neuronal excitation with all of these toxins can result in a tingling sensation, muscle spasms, agitation, seizures, cardiac arrhythmias and death potentially within an hour of exposure (White *et al.*, 1995; Spencer, 2000).

Pyrethrins, natural organic esters isolated from the flower of the *Chrysanthemum cinerariaefolium*, have

natural insecticidal properties as a result of their ability to bind to  $\text{Na}^+$  ion channels and slow their inactivation. These natural insecticides have a rapid knockdown effect of the insect followed by recovery. Synthetic analogs, pyrethroids, were developed to increase the insecticidal potency. Because of their relatively rapid metabolism, pyrethrins and pyrethroids are often marketed with synergists such as MGK-264 or piperonyl butoxide to inhibit enzymes which degrade them, thereby prolonging their insecticidal action. Today, pyrethroids are common ingredients in flea and tick spot-on products and sprays. Examples include permethrin, phenothrin, tetramethrin, fenvalerate, cyhalothrin, cyfluthrin, cypermethrin, cyphenothrin and etofenprox (Volmer, 2004; Merola and Dunayer, 2006).

Fish and cats represent the most susceptible animals to pyrethroids. Fish typically die acutely when exposed to pyrethroids. Cats are most commonly presented to the veterinarian as a result of exposure to the highly concentrated (45–65%) spot-on permethrin products labeled for use in dogs. Within 12–18h of application or accidental exposure, affected cats will exhibit hyperesthesia, generalized tremors, hyperthermia, seizures and possible death. The prognosis for complete recovery, however, is excellent with appropriate care. Seizures should be controlled with diazepam, barbiturates, inhalant anesthetics or intermittent propofol (caution regarding including Heinz body formation in cats with propofol). Tremors should be controlled with slow IV injection of methocarbamol (55–220mg/kg) to effect. Once severe tremors and seizures have been treated, decontamination with a warm bath using a liquid dishwashing detergent is indicated. Thermoregulation is critical. Hyperthermia can arise with continued tremors and seizures and could result in life-threatening disseminated intravascular coagulation. Hypothermia can ensue following a bath and can enhance or prolong the toxicosis. Supportive care with IV fluids can help protect the kidneys from myoglobinuric damage. Other species may have dermal hypersensitivity reactions or local paresthesia effects from topical application of pyrethroids. Steady resolution with this clinical picture is expected once the dermal residue is removed post bathing (Volmer, 2004; Hansen, 2006).

## Potassium channels

Potassium channels are largely involved with repolarization of the neuron following  $\text{Na}^+$ -induced depolarization. The opening of these potassium channels is voltage dependent and allows  $\text{K}^+$  to leave the cell, thereby restoring the resting potential of the axonal membrane. Several subtypes of potassium channels exist, the details of which exceed the scope of this text. Clinical effects of potassium channel-blocking toxicants

or adverse effects of similarly acting therapeutic agents depend on the type of  $K^+$  channel and location. A potent example of a potassium channel blocker in veterinary medicine is 4-aminopyridine (4-AP; Avitrol®). Predominantly used as an avicide, 4-AP ingestion by birds results in rapid onset of seizures and death (Schell, 2004). Although its classification as a 1A antiarrhythmic indicates it primarily blocks  $Na^+$  channels, quinidine, derived from the *Cinchona* tree, also blocks potassium channels. Depression, confusion and seizures are possible with quinidine overdose (Plumb, 2008). Classified as a class III (potassium channel blocker) antiarrhythmic, amiodarone also has sodium and calcium channel blocking effects. Adverse effects include bradycardia and hypotension and potentially hepatopathy but not primary nervous system effects. Sotalol, also a class III antiarrhythmic, exerts its pharmacological effect via blockade of cardiac potassium channels. It also has beta-blocking properties. Like amiodarone, adverse effects are primarily cardiac (bradycardia and hypotension), but nervous system effects secondary to hypoglycemia are also possible (Plumb, 2008).

### Chloride channels

As opposed to sodium and potassium channel neurotoxins, relatively few toxins have been identified that affect the chloride channel. Chloride ions can diffuse passively down their concentration gradient out of the neuron. Chloride ion channels regulate the entrance of chloride into the neuron and affect the membrane potential as a result. Normal resting membrane potential is 270mV. Threshold potential, the potential at which an action potential is propagated, is roughly 250mV. Membranes can be hyperpolarized by allowing entrance of negative chloride into the neuron and making it more difficult to reach threshold potential. Both benzodiazepines and barbiturates mediate their effects via GABA<sub>A</sub> receptors which are chloride ionophore complexes (Crystal and Schaumburg, 2000). Minute details regarding binding sites, duration and frequency of chloride channel opening effected by barbiturates and benzodiazepines have been extensively studied (Hobbs *et al.*, 1996; Crystal and Schaumburg, 2000; Maytal and Shinnar, 2000), yet the exact mechanism remains unclear. Ultimately, the two classes of drug inhibit excitatory neurotransmission by increasing chloride conductance into the neuron.

The use of potassium bromide for chronic management of idiopathic epilepsy in veterinary patients is based on the competition of the bromide ion with chloride ions for transport across cell membranes. The therapeutic action relies on hyperpolarization of the neuronal membrane and a decrease in the propagation of epileptic discharges. Bromide blood levels should be monitored routinely in treated animals and particularly in those

animals exhibiting signs of bromide toxicity. Neurologic signs consistent with a bromide toxicosis include ataxia, tremors and sedation to the point of stupor in veterinary species (Plumb, 2008). Headache, mood alterations, hallucinations, speech abnormalities and visual disturbances have also been reported with human bromism (Spencer, 2000). Because bromide has a longer half-life than chloride, the latter is preferentially excreted by the kidney. In animals with a deficiency of dietary salt, the half-life of bromide is prolonged, enhancing the chances of neurotoxicity. Conversely, the epileptic patient with a high dietary salt intake may have seizure activity that is poorly managed with potassium bromide treatment (Plumb, 2008).

## OTHER MECHANISMS OF NEUROTOXICITY

Knowledge of the nervous system is continually expanding. Whereas the mechanisms of action of neurotoxins discussed thus far have been simplified to affect one neurotransmitter or ion channel, the reality is likely far more complex. Additionally, clinicians should be aware of metabolic factors that can exacerbate neurotoxins. Examples of these factors include acidosis, hypoglycemia and hepatic encephalopathy. Increased carbon dioxide, which readily diffuses across the blood-brain barrier, can cause narcosis by affecting neurotransmitters as well as increasing intracranial pressure. Compensation for hypercapnea with hyperventilation is the reason that metabolic acidosis is less likely to contribute to encephalopathy than respiratory acidosis (Dewey, 2008). Monitoring of acid-base status, volume restoration, correction of metabolic acidosis with sodium bicarbonate as needed (Plumb, 2008) and assisted ventilation when necessary are means by which these potential complications can be minimized.

Hypoglycemia can be a primary effect of neurotoxins as with sulfonylurea medication overdoses or xyli-tol ingestion in dogs (Meadows, 2011) or a secondary effect with increased glucose utilization in the patient with refractory seizures. Signs of neuroglycopenia occur when blood glucose goes below 45mg/dl and include weakness, ataxia, collapse, restlessness, tremors, seizures, blindness and potentially changes in behavior (Podell, 2000; Nelson, 2009). Hypoglycemia can be corrected with administration of intravenous dextrose to effect (Meadows, 2011).

Finally, hepatic encephalopathy can result in neurotoxicity as a result of the inability of the liver to clear the body of toxins, namely ammonia, and the brain's sensitivity to it. Alterations of serotonin, GABA and glutamine and stimulation of NMDA and

benzodiazepine receptors ensue which are responsible for potential disorientation, gait disorders, behavioral changes and/or seizures (Watson and Bunch, 2009). Treatment with lactulose to lower blood ammonia, appropriate antibiotics, supportive care and management of seizure activity are indicated (Webster, 2011).

## CONCLUSIONS

The health of the nervous system, and largely that of the individual, relies on the system's structural and functional integrity. From specialized nerve cells and anatomic structures to axonal transport, myelination, neurotransmitter synthesis, storage, release, binding and degradation as well as the regulation of action potentials,

maintenance of nervous system integrity is a complex task requiring significant energy expenditure. This functional complexity and structural specialization provide a plethora of targets for neurotoxicant action. The scope of this chapter precludes a detailed discussion of every known neurotoxicant. Many more mechanisms exist by which neurotoxicants exert their effects. Table 14.3 provides a comprehensive, yet certainly not exhaustive, list of neurotoxicants grouped by mechanism or site of action, where known.

The extent to which a neurotoxicant exerts its effects depends on a variety of factors including dose, species, age, genetics, underlying diseases, drug therapy, diet, stress and concurrent toxicoses. Veterinary practitioners should be aware of these underlying susceptibilities and the most common mechanisms by which neurotoxicants act so that toxicoses may be avoided or appropriately diagnosed and successfully treated when they do occur.

TABLE 14.3 Neurotoxicants arranged by mechanism of action (Beasley, 1999; Spencer, 2000; Burrows and Tyrl, 2001; Schulze, 2002; Plumlee, 2004)

### Mediation by acetylcholine

*Amanita muscaria* (minute amount)  
Anatoxin-a  
Anatoxin-a<sub>(s)</sub>  
*Atropa belladonna*/atropine  
Botulism  
Carbamate insecticides  
*Clitocybe* mushrooms  
*Conium maculatum* (poison hemlock)  
*Datura* (jimsonweed)  
*Gymnocladus dioica* (Kentucky coffee tree)  
Imidacloprid  
*Inocybe* mushrooms  
*Lobelia* (Indian tobacco)  
Nicotiana  
Organophosphorous insecticides  
Slaframine

### Mediation by norepinephrine

Albuterol  
Atipamezole  
Atomoxetine  
Brimonidine  
Clonidine  
Doxazosin  
Medetomidine  
Metoprolol  
Monoamine oxidase inhibitors (MAOIs)  
Propranolol  
Phentolamine  
Phenoxybenzamine  
Sotalol  
Tamsulosin  
Terazosin  
Tricyclic antidepressants (TCAs)  
Venlafaxine  
Yohimbine  
Xylazine

### Sympathomimetics/catecholamines

Amphetamines  
Cocaine  
Ma huang (*Ephedra*)  
MDMA/"Ecstasy"  
Monoamine oxidase inhibitors (MAOIs)  
Phenylpropanolamine  
Pseudoephedrine  
Tricyclic antidepressants (TCAs)

### Mediation by serotonin

Amphetamines  
*Griffonia*/5-HTP supplements  
Lysergic acid diethylamide (LSD)  
MDMA/"Ecstasy"  
*Phalaris* (Canary grass poisoning)/tryptamine alkaloids  
*Psilocybe* mushrooms  
Selective serotonin reuptake inhibitors (SSRIs)  
Tricyclic antidepressants (TCAs)  
Tryptophan  
Venlafaxine

### Mediation by glycine

*Calycanthus* (bubby bush)  
Strychnine  
Tetanus  
Tremorgenic mycotoxins (penitrem A and roquefortine)

### Mediation by GABA

Avermectins  
Baclofen  
Barbiturates  
Benzodiazepines  
Bicuculline  
*Cicuta* (water hemlock)  
Cyclodiene organochlorines  
Fipronil  
Flumazenil  
Isoniazid

(Continued)



TABLE 14.3 (Continued)

<b>Mediation by glutamate/aspartate</b>	Non-protein nitrogen
Amantadine	NSAIDs
+/- <i>Centaurea solstitialis</i> (yellowstar thistle)	Pyrrolizidine alkaloids:
Domoic acid	<i>Senecio</i>
Ketamine	<i>Crotalaria</i>
<b>Mediation by histamine</b>	<i>Heliotropium</i> (giant hog weed)
Brompheniramine	<i>Amsinckia</i> (fiddle neck, tarweed)
Chlorpheniramine	<i>Symphytum</i> (comfrey)
Cetirizine	<b>Stimulating cannabinoid receptors</b>
Cimetidine	<i>Cannabis sativa</i> (marijuana)
Cyproheptadine	<b>Mediation of opioid receptors</b>
Dimenhydrinate	<i>Aesculus hippocastanum</i> (horse chestnut)
Diphenhydramine	Buprenorphine
Famotidine	Butorphanol
Fexofenadine	Codeine
Hydroxyzine	Fentanyl
Loratadine	Heroin
Ranitidine	Hydromorphone
Scombroid fish poisoning	Loperamide
<b>Effect on sodium channels</b>	Meperidine
Aconitine	Morphine
Batrachotoxin	Naloxone
Brevetoxins	Oxymorphone
Ciguatoxin	Oxycodone
Funnel web spider toxins	Tramadol
Grayanotoxins	<b>Multiple/miscellaneous MOAs</b>
Local anesthetics	<i>Ageratina</i> (white snakeroot): tremetone
Pyrethroids	<i>Astragalus/Oxytropis</i> (locoweed): miserotoxin
Saxitoxin	Bovine bonkers/ammoniated feed): 4-methylimidazole
$\alpha$ and $\beta$ scorpion toxins	<i>Brunfelsia</i> (yesterday, today and tomorrow plant): brunfelsamidine
Sodium intoxication	<i>Cycas</i> (sago palm): $\beta$ -methylamino-L-alanine
Tetrodotoxin	5-Fluorouracil/1080
Veratridine	Lead
<b>Effect on potassium channels</b>	<i>Macrozamia</i> (cycad palms): $\beta$ -oxalylamino-L-alanine
4-Aminopyridine	Mercury
Amiodarone	Methylxanthines, <i>Guarana</i> (adenosine receptor antagonists)
Quinidine	Phenothiazines (anticholinergic, antihistaminic, $\alpha$ -adrenergic blocking effects)
Sotalol	<i>Sorghum</i> (Sudan grass): $\beta$ -cyanoalanine
<b>Effect on chloride channel</b>	<i>Stipa</i> (sleepy grass): ergot alkaloids
Avermectins	<b>Metabolic</b>
Barbiturates	Carbon monoxide
Benzodiazepines	Cyanide
Picrotoxin	Zinc phosphide
Potassium bromide	<b>Thiamine deficiency/thiaminase excess</b>
<b>Producing acidosis</b>	<i>Equisetum</i>
Aspirin	<i>Pteridium</i> (bracken fern)
Ethanol	Raw fish diet
Ethylene glycol	Excess dietary sulfur
Metaldehyde	<b>Uncoupler of oxidative phosphorylation</b>
Methanol	Bromethalin
<b>Causing hypoglycemia</b>	Hexachlorophene
<i>Xanthium</i> (cocklebur)	Trialkyltin
Sulfonylureas	<b>Unknown etiology/MOA</b>
Xylitol	Desert spike
<b>Producing hepatic encephalopathy</b>	Diethyltoluamide (DEET)
Acetaminophen	Essential oils
Alsike clover (horses)	Hydrocarbons
Blue-green algae (microcystins)	Indoxacarb
<i>Cycas</i> (sago palm): cycasin	Macadamia nuts
<i>Lantana</i>	Nitrofurantoin
<i>Macrozamia</i> (cycad palms)	Tick paralysis
D,L-Methionine	

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## Respiratory toxicity

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### INTRODUCTION

The respiratory system is relevant in toxicology as a target of toxic effects, and also as a major route of absorption of inhaled gases and atmospheric particles. If inhalation and subsequent absorption reach a toxic threshold, inhaled chemicals have the potential to produce lung disease. If the chemical is trans-located to other organs, disease can be produced in those organs. As a highly perfused organ system, the respiratory system is also a potential target for toxic chemicals in the blood circulation, irrespective of the route of absorption. Inhalation toxicology refers to the respiratory system as a route of exposure. In contrast, respiratory toxicosis refers to respiratory organ toxicosis. The environments in which animals live are directly relevant to respiratory toxicosis (Pickrell, 1991). Different species may have anatomical features that lead to specific patterns of deposition and absorption of chemicals from the air, resulting in species variations in adverse effects (Morris *et al.*, 1986).

To understand respiratory toxicosis we need understanding of (1) toxicant dose delivered to specific sites of the lung and (2) the effects of toxicants on respiratory system tissues under conditions of acute and chronic exposures. Many lung diseases have their beginnings with increases in oxidative lung burden. There is strong evidence that oxidative lung burden may favor development of lung diseases such as bronchitis, asthma and interstitial lung disease (Witschii and Last, 2001).

Advances in lung toxicology are augmented by interdisciplinary collaboration between anatomists, physiologists, immunologists, pathologists, molecular biologists, epidemiologists, clinicians and many other shared disciplines. Adaptation of pulmonary function

tests to animals has led to the comparison of human and animal data, generally providing more robust conclusions compared to data from single species. Recent molecular measurements have brought home how closely tied many of these diseases are to specific protein changes. Such molecular analyses help to pinpoint specific molecular reactions to specific cell types. In addition, they help us describe the more relevant influence of each cell on the others in the near neighborhood. Tissue culture, organ minces and *in vivo* animal exposure studies, such as broncho-alveolar lavage washings, further elucidate these interactions.

### THE UPPER AIRWAYS

#### Normal function

The nasopharynx is lined by pseudostratified columnar ciliated epithelium with goblet cells (Dungworth, 1993). Abundant lymphoid nodules are present in the submucosa. The eustachian tubes extend from the middle ear to the nasal cavity. Many animals, including horses and small laboratory rodents, are obligate nose breathers. Other animals can breathe through either their nose or mouth.

The mucous membranes of the nasal passages produce secretions from serous and mucous glands, which produce mucopolysaccharides and mucoprotein, and goblet glands, which produce similar, but sulfated, secretions. Ciliated epithelium covers a capillary net providing a rich blood supply. Mucous secretions protect the mucous membranes, and can retain particulate matter and



substances that dissolve in the mucous. It also allows for rapid heat transfer between the capillary net and the air (Taylor, 1974). Beating of cilia propels mucous and allergens, pathogens and toxicants that become entrapped in the mucous towards the glottis, from where it is shunted to the digestive tract by swallowing (Dungworth, 1993). Effective mucociliary clearance depends on coordinated secretions by the goblet cells, sub-mucous glands and serous secretory cells to form an upper mucous layer underlying a more aqueous layer, which allows the upper mucous layer to be transported by cilia (Corcoran, 2010). Non-ciliated (Clara) epithelial cells have P-450 monooxygenase systems that can metabolize xenobiotics, and they can produce inflammatory compounds such as prostaglandins. The mucociliary clearance pathway can adapt to changing environmental conditions, and forms a line of defense against a variety of airborne insults.

Upper conducting airways (bronchi, progressively smaller generations of bronchioles and terminal bronchioles) are lined with pseudo-stratified columnar epithelial cells with a few goblet cells interspersed. Their support tissue contains fibrous connective tissue and cartilaginous plates (Dungworth, 1993). Animals adapted for strenuous physical activity have larger diameter airways to accommodate additional airflow. As bronchioles become progressively smaller, resistance to airflow increases. In these small conducting airways, diseases reflecting altered airflow resistance are said to be diseases of small airways or small airway resistance. As the airways narrow, the epithelium changes from pseudo-stratified ciliated columnar cells with goblet cells to simple columnar epithelium, reflecting the form, function and stresses of these smaller airways. The smallest bronchioles may collapse near the end of expiration, unless there is sufficient collateral circulation to keep them open.

Upper airways (bronchi and bronchioles) have bronchial associated lymphoid tissue; this tissue is similar to gut-associated lymphoid tissue (Dungworth, 1993). Bronchial associated lymphoid tissue is responsible for local immunity in the lung.

### Toxic diseases of the nasal passages

Epistaxis is a typical clinical effect associated with anti-coagulant pesticide toxicosis in animals (Berny, 2007). High doses of ethylene glycol (>10mg/kg) have produced bovine epistaxis and hemoglobinuria (Crowell *et al.*, 1979). The likelihood of equine exercise-induced pulmonary hemorrhage is increased by airborne reactive oxygen substances (ROS), which can destroy the capillary barrier and reduce vasoactive nitric oxides (Manohar *et al.*, 1993; Mills and Higgins, 1997). Rattle snake envenomation is another toxic cause of epistaxis in

horses (Dickinson *et al.*, 1996). These horses have fever, tachycardia, cardiac arrhythmia, thrombosis and hemorrhage. Since horses are often bitten on the nose and are obligate nose breathers, the nostril may swell and must be kept patent.

### Toxic diseases of the conducting airways

Allergic rhinitis that clinically resembles hay fever in humans can occur in horses, cats and dogs (Dungworth, 1993). Nasal granuloma is a chronic form of allergic rhinitis, characterized by degranulation of mast cells and infiltration by eosinophils. Horses with chronic obstructive pulmonary disease have high transpulmonary pressures (Ammann *et al.*, 1998). Constant exposure to dust through litter use or upper respiratory tract infections were blamed as likely causes of feline upper airway inflammation. However, only certain cats become clinically ill, suggesting varying degrees of hypersensitivity to stimulating antigens in cat populations (Dye *et al.*, 1992). Cattle, especially the channel island breeds, and occasionally sheep have a seasonal allergy that resembles this condition. Cattle are believed to be allergic to pollen antigens. Histologically, the epithelium is hyperplastic, eroded and/or infiltrated with eosinophils.

A canine model of nasal congestion and allergic rhinitis has been described (Tiniakov *et al.*, 2003). A very high prevalence of inflammatory gastrointestinal tract problems in brachycephalic dogs that presented with upper respiratory problems was observed clinically, endoscopically and histologically. Some histologically observed inflammatory lesions were not macroscopically visible at endoscopy. Respiratory and digestive signs correlated closely in French bulldog males and heavy brachycephalic dogs (Poncet *et al.*, 2005). It is thought that systemic inflammatory disease can lead to secondary pulmonary infiltration and inflammation (Benedice *et al.*, 2003).

Moderate smoke inhalation injury to the upper airway is usually independent of temperature damage; after as little as 1 hour, endothelin-1 in cells that line the airway increase (Cox *et al.*, 2001). Such changes may contribute to airway inflammation, mucus secretion, pulmonary hypertension, increased airway resistance and decreased lung compliance, in an ovine model of smoke inhalation injury (Cox *et al.*, 2001).

## THE GAS EXCHANGE REGION

### Normal function

Pulmonary parenchyma is divided into units of structure and function called acini (Dungworth, 1993). An acinus

is the gas exchange unit of the lung and of pulmonary parenchyma. The acinus includes all branches of respiratory bronchioles, alveolar ducts and alveoli associated with ventilation; in addition, it contains the vasculature associated with perfusion. Lobules are many acini grouped together and surrounded by connective tissue; in cattle, sheep and horses they form lobules visible at autopsy. In other species lobules are less well outlined. Cells most important to the gas exchange are epithelium (type II and type I epithelial cells). Fibroblasts provide connective tissue and structural support for the alveolus; vascular endothelial cells line the pulmonary capillaries.

### **Ventilation**

Oxygen-rich inhaled air flows through the conducting airways to reach the acini. Oxygen and carbon dioxide are exchanged between capillary blood and air across the alveolar epithelium and vascular endothelium by diffusion along the gas concentration gradient. Carbon dioxide-enriched air is expelled upon expiration. Total lung capacity (TLC) refers to the volume of air in inflated lungs. Some volume of air remains in the lungs following maximum expiration, known as the residual volume (RV). The volume of air moved in and out during breathing is called the tidal volume (TV), while vital capacity (VC) refers to the volume of air moving in and out of the lungs during maximum inhalation and expiration. Oxygen delivery to the blood can be increased by increasing the tidal volume, the respiratory rate or the oxygen concentration in the inspired air. Tidal volume has a fraction in the conducting airways that does not exchange gas, known as the dead volume (West, 2000a). Anatomic dead space refers to the volume of the conducting airways to where oxygen becomes diluted (Fowler's method). Physiologic dead space refers to the portions of the airways that do not contribute to the exchange of carbon dioxide (Bohr's method; West, 2000a). As the breathing pattern becomes shallower and more rapid, the fraction of the non-contributing dead volume in each breath increases. Although we often assume that all regions of the lung are ventilated equally, positional differences are seen in humans. Such differences are minimized when humans are in the supine position. In dogs, the anterior main bronchi receive more ventilation than do the rear ones.

The water solubility of gases influences how deeply they penetrate into the airways and terminal lung structures. Highly water soluble gases, such as SO<sub>2</sub>, do not penetrate deeply. Less water soluble gases, including NO<sub>2</sub>, ozone, CO and H<sub>2</sub>S, penetrate to the deepest lung structures.

Ozone and oxides of nitrogen and sulfur were modeled for absorption throughout the respiratory tract (Tsujino *et al.*, 2005). All three gases had higher

concentration in the airways. For example, ozone was 3–12 times higher at the 5th generation bronchus. Sensitivity analysis indicated that tidal volume, respiratory rate and surface area of the upper and lower airways significantly affected the results. Kinetics of inhaled gaseous substances vary substantially among animals and humans, and such variations are, at least partially, the result of anatomical and physiological differences in their airways.

### **Perfusion**

There are two different types of blood supply to the lung, nutrient vessels that provide nutrition for the lung and pulmonary vessels that specialize in exchanging alveolar oxygen onto the hemoglobin to be carried to the target organs (West, 2000a). The capillaries are just large enough to admit erythrocytes and they are very short. Blood flows around the alveoli as a sheet, and the network of capillaries is said to resemble a miniature underground parking garage.

### **Diffusion**

For some gases for which the blood has a large carrying capacity, such as carbon monoxide, the rate of uptake into the blood is limited by the blood–gas barrier. Thus, the gas exchange is described as diffusion limited. For certain other gases, such as nitrous oxide, which do not bind to or are taken up by the red blood cells, uptake is not limited by diffusion, but by the available blood volume provided by alveolar perfusion. These gases are described as perfusion limited. Oxygen takes the middle road so that its uptake is dependent on the blood–gas barrier characteristics (diffusion limited), as well as the blood volume (perfusion limited). If the alveolar wall becomes abnormally thick, for example when collagen is deposited in the interstitium or with the accumulation of interstitial fluid (edema), the oxygen uptake rate across the barrier is reduced. Alternatively, and more commonly, a failure to match ventilation with perfusion is the cause of poor gas exchange (West, 2000b). If a lung is heavily perfused with minimal ventilation because of a blocked airway, the amount of oxygen that can be exchanged is limited by the mismatch of poor ventilation with good perfusion. Alternatively, gas exchange is compromised if the lung is efficiently ventilated, but receives little or no perfusion. Both conditions are referred to as ventilation–perfusion mismatches.

### **Avian respiration**

There are many morphologic, physiologic and mechanical differences between the avian and mammalian respiratory systems (Brown *et al.*, 1997). In birds, the gas exchange unit is the parabronchus. The parabronchus

has no alveoli. The walls of the narrow passageways called air capillaries serve as the gas exchange surface. Inhaled air reaches the parabronchus via the upper air passageways. Oxygen is extracted as it moves along the air capillaries. After passing through the parabronchus the air is not expelled directly, but moves on to the air sacs. From there the air is moved back through the parabronchus before it is expelled. The parabronchus does not expand and contract like mammalian lungs. Instead, air movement is controlled by expansion and compression of the air sacs. The parabronchus–air sac system provides two opportunities to extract oxygen from inhaled air. In addition, counter flow between blood and air in the parabronchus provides an effective oxygen concentration gradient over a larger fraction of the gas exchange surface area. Partially oxygenated blood comes into close proximity to the air with the highest oxygen concentration, while air with lower oxygen pressure comes into close proximity to blood with the lowest oxygen concentration. Oxygen is extracted about twice as efficiently as in mammals, which is necessary for the increased oxygen demand associated with sustained flight. Air sacs and air membrane spaces provide a reduction in density to birds; reduced density is helpful in staying afloat on water. The increased gas exchange efficiency in birds makes them more susceptible to some inhaled toxicants, such as the fumes released from overheated non-stick cookware coated with polytetrafluoroethylene (Lightfoot and Yeager, 2008). This sensitivity can also be exploited as a very sensitive monitor of air quality. An example of this is the canary in the mine that predicts toxicity to the miners (Brown *et al.*, 1997; West *et al.*, 2006).

## GENERAL PRINCIPLES IN THE PATHOGENESIS OF LUNG DAMAGE CAUSED BY CHEMICALS

### Oxidant burden

Oxidant burden in the lung is frequently associated with airborne pro-oxidants such as nitrogen dioxide, sulfur dioxide, oxidants such as ozone, free radicals, tobacco smoke, or is caused by an overzealous defense by phagocytic cells (Pickrell *et al.*, 1987a; Pickrell and Mageed, 1995; Witschi and Last, 2001). Exposure to oxidants can lead to changes in lung structure and biochemistry (Pickrell *et al.*, 1987b; Witschi and Last, 2001). Pivotal roles have been established for superoxide, pro-oxidant peroxy nitrites, and hydroxyl radicals. Responses depend on the oxidant burden in combination with the glutathione or biological antioxidant concentration. With sufficient oxidant burden and depletion of glutathione, all lung toxins have an inflammatory disease component.

In the presence of significant oxidant burden, but in the presence of high levels of glutathione, lung defensive metabolic enzymes are activated. At intermediate glutathione levels, inflammation is activated using nuclear factor kappa beta. At lower levels of glutathione, mitochondrial enzymes are activated. The relation of programmed cell death (apoptosis) to mitochondrial enzyme activation is being investigated (Nel *et al.*, 2006).

### Toxic inhalant gases

#### Chlorine

Exposure to chlorine may originate from the manufacture of pulp, paper, plastics and chlorinated chemicals (Witschi and Last, 2001). Chlorine gas is irritating to the upper airways, and can cause hemoptysis, dyspnea, tracheobronchitis or even bronchopneumonia in animals inhaling sufficient concentrations. Peak symptoms are typically reached in 30–90 minutes. Patients only infrequently require more than monitoring and rudimentary support measures. Full recovery from such injuries is likely, but permanent loss of function is possible in cases of exposure to very high levels of chlorine (Winder, 2001). Humans exposed to chlorine may have a decreased vital capacity (limited forced expiratory volume) (Mehta *et al.*, 2005). This limitation is frequently reversible and less than that of seasonal allergic rhinitis (Shusterman *et al.*, 2004).

#### Ammonia

Animals and caretakers are frequently exposed to elevated levels of ammonia (NH<sub>3</sub>) gas in swine confinement facilities (Carson, 2004). At greater than 100 ppm, ammonia irritates eyes and respiratory membranes, increases the incidence and intensity of microbial or parasitic infections, and reduces growth rate. High levels of ammonia found in poultry houses have about the same level of toxicity to birds as they would to other animals (Brown *et al.*, 1997). Ammonia concentrations greater than 60 ppm cause kerato-conjunctivitis in broilers; reduced bacterial clearance and enhanced sensitivities to bacterial infections (Carson, 2004).

#### Anhydrous ammonia

Anhydrous ammonia (NH<sub>3</sub>) is injected from pressurized tanks into the ground as a fertilizer nitrogen source. It can be lethal to animals and humans if pressurized tanks are breached or large containers are spilled in transit (Carson, 2004). Anhydrous ammonia reacts with air moisture to form a vapor cloud which can either remain for several hours or disperse efficiently, depending on wind velocity and humidity. In animals and man, the eyes and upper respiratory tract are prime targets. If the air ammonia concentration is higher than 5000 ppm, it



can cause a fatal apnea or laryngeal edema. Survivors may be blinded by corneal lesions and sloughed epithelium. Removal of animals is important if the vapor cloud does not disperse rapidly. Supportive therapy may be curative in moderate exposures. In humans, loss of olfactory ability was associated with exposure to anhydrous ammonia, as well as a history of wheezing and asthma, and of flu-like illness (Snyder *et al.*, 2003).

### Carbon dioxide

Carbon dioxide (CO<sub>2</sub>) is well tolerated, even at concentrations as high as 5%. Higher concentrations stimulate the rate and depth of respiration (Carson, 2004). It is, however, being explored as a novel human stressor (Kaye *et al.*, 2004). It is an asphyxiant at extremely high concentrations (>40%). Because it is heavier than air, it collects in the lower portion of animal facilities (Carson, 2004). It is used as a euthanasia agent in some laboratory animal species.

### Carbon monoxide

Carbon monoxide (CO) is a product of incomplete combustion of hydrocarbon fuels. It has background levels of about 0.02 ppm in rural areas, 13 ppm in urban areas and 40 ppm in areas of high urban traffic. Improperly vented or adjusted heaters, and fires, especially those burning more coolly, are frequent sources of increased CO.

Carbon monoxide binds about 250 times as tightly to hemoglobin as oxygen, forming carboxyhemoglobin (COHb). Oxygen is displaced by CO, limiting the ability to take up oxygen and give off carbon dioxide in the lungs. Because of their high respiratory exchange efficiency, birds are unusually sensitive to carbon monoxide (Brown *et al.*, 1997). They often require relatively more oxygen per unit body weight because they have smaller body sizes and more active metabolisms.

Measurement of COHb is diagnostic of CO intoxication (Carson, 2004). In humans, <3% COHb is considered normal; 6–8% causes drifting of attention; 10–20% headaches; 20–30% dizziness; 30–60% tachypnea, tachycardia and confusion; and 60% fatality. Birds respond more acutely than mammals, and canaries have been used as sentinels for miners (Brown *et al.*, 1997; Carson, 2004). Treatment requires, at a minimum, fresh circulating air; 100% oxygen may be lifesaving. Prognosis depends on the amount of COHb and the hypoxic brain damage. Pulmonary function should be monitored for at least 2 weeks, and in some cases 6 weeks.

### Methane

Methane (CH<sub>4</sub>) becomes an asphyxiant at >85%; it is an explosion hazard at 10–15%. It is substantially lighter than air and will flow above water in a swamp (Carson, 2004).

### Hydrogen sulfide

Since hydrogen sulfide (H<sub>2</sub>S) is insoluble in water, it may expose the deepest recesses of the lung. At 50–150 ppm level H<sub>2</sub>S causes pulmonary edema (Carson, 2004). *In vitro*, H<sub>2</sub>S induces apoptosis of aorta smooth muscle cells, regulated by mitogen-activated protein kinase (ERK MAPK) that activates caspase-3 (Yang *et al.*, 2004). H<sub>2</sub>S is less toxic to birds than to other animals; 2000–3000 ppm will change respiratory rate and depth, while 4000 ppm will kill them in about 15 minutes. The mechanism may relate to the greater gas exchange efficiency of parabronchi (Brown *et al.*, 1997).

Hydrogen sulfide's ability to paralyze the respiratory tract is its greatest danger to animals and humans (Carson, 2004). Above 500–2000 ppm, mammals are said to take the second, but not the third, breath. Above 500 ppm, H<sub>2</sub>S begins to cause permanent neurologic damage. Data from rats trained to run a reversed contingency maze suggested that H<sub>2</sub>S may impair learning by increasing the animals' susceptibility to interference from irrelevant stimuli (Partlo, 2001).

Hydrogen sulfide is readily detectable as a rotten egg smell. Humans and presumably animals can detect hydrogen sulfide at 0.025 ppm (Carson, 2004). Above 200 ppm, however, hydrogen sulfide paralyzes the olfactory apparatus so it may not be detectable by smell. Higher concentrations seem to paralyze smell more rapidly. Thus, animals or humans may have only a very brief instant to smell hydrogen sulfide at high concentrations; it is dangerous to ignore the smell because its duration can be so brief.

Hydrogen sulfide is heavier than air and insoluble in the water of manure pits (Carson, 2004). Thus it can exist as bubbles in swine manure pits inside hog houses, ready to be released and expose animals and workers on agitation. Taking appropriate steps to protect rescuers, exposed people or animals not breathing should be dragged outside. If breathing is not re-established spontaneously, artificial respiration should be applied until spontaneous respiration returns.

### Nitrogen dioxide and ozone

Nitrogen dioxide (NO<sub>2</sub>) is considered with ozone (O<sub>3</sub>), because NO<sub>2</sub> is a pro-oxidant and O<sub>3</sub> an oxidant. In agriculture, NO<sub>2</sub> can come from silage or relatively airtight silos, where it is usually found towards the top (Carson, 2004). Indoor air NO<sub>2</sub> and O<sub>3</sub> can come from second-hand cigarette smoke. Nitrogen dioxide exposure from newly opened silage bags may modestly affect hungry cattle if exposure levels are unusually high (i.e., in large confined animal feeding operations dairies), but usually they are more likely to affect caretakers in upright silos. Nitrogen dioxide has low water solubility, and can pass through the upper airway and permanently damage pulmonary parenchyma where residence times are



longer. At ambient NO<sub>2</sub> (2–3 ppm) there is little damage or clinical signs. At higher levels, for example 20 ppm, it induces lung edema. Animals develop coughing, some fluid in the lungs, death of type I epithelial cells, coalescing alveoli and an increased collagen production, but no morphologic evidence of fibrosis (Gregory *et al.*, 1983; Pickrell *et al.*, 1987a; Mauderly *et al.*, 1987; Carson, 2004). Animals that die at varying times after exposure have evidence of pulmonary edema and emphysema.

Birds are unusually sensitive to NO<sub>2</sub> and O<sub>3</sub>, depending on exposure level. Caged pet birds may be sensitive to second-hand cigarette smoke, especially in the presence of heating/combustion sources. Newly hatched chicks die after 5 days' exposure to 1–4 ppm O<sub>3</sub>; exposure to 0.3–0.7 ppm O<sub>3</sub> causes pulmonary hemorrhage in these chicks (Brown *et al.*, 1997). Pulmonary hemorrhage in mammals is typical of acute high-level oxidant exposure of a somewhat more intense nature than that associated with pulmonary edema (Pickrell *et al.*, 1987a).

## Particle size and clearance

### Particle size and deposition mechanisms

Inhaled aerosol particles are frequently of multiple sizes (polydispersed). Particles of different aerodynamic sizes deposit in different anatomic areas of the mammalian and avian respiratory tracts. In mammalian lungs, particles with aerodynamic diameters of 5–30 micrometers mostly deposit by inertial impaction in the nasopharyngeal region because they do not follow curving airflow well (Witschi and Last, 2001). Inertial impaction in bird respiratory tracts is influenced by flow velocity and Reynolds numbers (Re) (trachea 100 cm/sec at Re = 700; primary bronchi 130 cm/sec and Re = 600; narrowed primary bronchus 200 cm/sec and Re = 550). The expected sites of inertial impaction in birds are similar to those in mammals (Brown *et al.*, 1997).

Slightly smaller particles (1–5 micrometers in aerodynamic diameter) mostly deposit by gravitational sedimentation in the conducting airways – the trachea, bronchi and bronchioles – as airflow speed progressively slows down towards the airway terminals. In the human lung, sedimentation is an important mechanism for deposition at sizes greater than 0.2 micrometers in diameter (Witschi and Last, 2001). In birds, deposition probability is a product of (residence time)(aerodynamic diameter). In parabronchi, where flows are as low as 3 cm/sec (Re = 2), and residence times may be up to 1 min for complete change of air sac volume at rest, deposition probabilities can be quite high (Brown *et al.*, 1997).

Particles <1 micrometer in aerodynamic diameter have a good probability to deposit by Brownian diffusion when their random movement causes them

to bump into wet surfaces (Witschi and Last, 2001). Diffusion, like sedimentation, is most important where residence times are the longest. The probability of deposition by diffusion increases with decreasing particle size, so that deposition of particles with aerodynamic diameters of less than 0.5 micrometers is largely by diffusion. Breathing pattern can be important. During exercise particles acquire higher velocity, momentum and inertia that increase impaction deposition higher up the respiratory tract. Alternatively, breath holding causes more deposition by gravitational sedimentation and diffusion.

### Particle clearance

Particle clearance in mammals is important to defense of the lung. Rapid clearance lessens exposure time and extent of injury. Clearance of intact particles depends heavily on pulmonary alveolar macrophages and the mucociliary escalator. Alveolar macrophages phagocytose particles, then migrate to the bronchi and trachea for clearance. Ultrafine particles that enter the alveolar interstitium may be cleared at a much slower rate (Shinohara *et al.*, 2010). Particles can leave the lung and enter the vascular system, causing a risk to organs downstream from the lung (Nemmar *et al.*, 2001; Borm *et al.*, 2006). Alternatively particles may be soluble in aqueous media and can be dissolved in mucus. Fine and ultrafine (nano) particles with much of their mass in contact with the surface are especially subject to such dissolution. Factors affecting this solubility are quite complex; they include but are not limited to composition of the aqueous fluid, particle size, particle chemical composition, steepness of concentration gradient, curvature, agglomeration and aspect ratio (Nemmar *et al.*, 2001; Borm *et al.*, 2006). Some ultrafine particles that are deposited in the nasal mucosa can be transported to the brain via the olfactory bulb (Oberdörster *et al.*, 2004).

Particle solubility and chemical reactivity are influenced by the physical–chemical environment. Bicarbonate in lung simulant fluid, for example, can enhance solubility in particles moderately soluble in aqueous media. To obtain relevant data solubility and reactivity should be measured in an environment as near to that of the lung environment as possible. This can sometimes be accomplished with *in vitro* conditions closely simulating biological solutions (Powers *et al.*, 2006). Solubility is thought to be a function of chemical activity, specific surface area, radius and curvature, agglomeration and specific chemicals that are adsorbed to the NSP (Borm *et al.*, 2006). It is important to consider not just solubility in water, but in fluids that bear significant resemblance to that of the epithelial lining fluid (ELF). Epithelial lining fluid is the fluid that lines the epithelial cells in the respiratory tract.

A volume of 40–100 ml is predicted for mature humans. The pH varies from 6.9 at the end of inspiration to 7.5 at the end of expiration. Bicarbonate is a major buffer, and the pH variation is caused by varying CO<sub>2</sub> during expiration (Langmuir, 1965). Bicarbonate in lung simulant fluid increases the solubility of magnesium oxide (MgO) (Pickrell *et al.*, 2006). Stoichiometry of the likely chemical species suggests a conversion of MgO to magnesium hydroxide (Mg(OH)<sub>2</sub>) in aqueous media, and a subsequent conversion to the hydrated carbonate nesquehonite.

In chickens, small dust particles were found trapped in the respiratory epithelial cell trilaminar surfactant (Brown *et al.*, 1997). No particles were found in the vascular system, kidneys and heart, suggesting that at least those particles did not enter the vascular system. After 1 hour 54% of the particles remained, while after 36 hours approximately 36% remained, suggesting that appreciable clearance took place. The mechanism of the clearance is not completely clear. However, parenchymal respiratory epithelial cells could have transported particles toward the pulmonary interstitium. Particles in the mammalian lung too small to be efficiently phagocytized were also transported into the lung's interstitium. In bird lungs, there was a virtual absence of phagocytes; the absence may have caused the epithelial cells to take on a larger role. Following experimental introduction of Sephadex or Freund's adjuvant, phagocytes with physiological features similar to macrophages can enter the bird's pulmonary spaces.

Birds living in dusty environments – kiwis near the desert with abundant sand and poly dispersed loose dust, or birds living near or flying over volcanic ash – show significant pathology and signs after only short exposures to small dust particles. Confined animal feeding operations with poultry raised in high population densities often show reduced production and pathology at necropsy (Brown *et al.*, 1997).

## LUNG TO INJURY

### Acute responses to lung injury

#### Airway reactivity

Mammalian large conducting airways are surrounded by bronchial smooth musculature (BSM) (Witschi and Last, 2001). The muscle tone is modulated by the autonomic nervous system. Reflex contraction occurs after stimulation by irritants. Bronchoconstriction can also be provoked by cholinergic drugs. This stimulation is used in diagnosis as a basis to assess the "twitchiness" of BSM – at how low a level the bronchi can be induced to constrict.

These agents bind to receptors and trigger increased intracellular cyclic monophosphates – adenosine and guanosine, respectively (cAMP and cGMP). Histamine, prostaglandins, nitric oxide and leukotrienes also influence bronchoconstriction.

#### Pulmonary edema

Pulmonary edema is exudative, and it indicates acute lung injury (Witschi and Last, 2001). Edema is associated with leakage or interruption, as well as thickening of the alveolar capillary membrane. Edema fluid creates a thicker diffusion barrier, and contributes to a mismatch of ventilation to perfusion. The mismatch limits the rate of oxygen exchange from the air of the alveoli to the blood of the alveolar capillaries, even if the alveolar capillary unit is normal in all other respects. Frequently, very intense pulmonary edema will include some evidence of small pinpoint pulmonary hemorrhage. Even brief edema may damage or denude epithelium, or endothelium, or both.

Heart failure is often associated with cardiogenic lung edema, as demonstrated by a case of fatal *Taxus* (Japanese yew) poisoning in horses with pulmonary congestion, hemorrhage and edema (Cope *et al.*, 2004), as well as calves given *Nerium oleander* (Oleander) clippings, which developed tachycardia and pulmonary edema (Galey *et al.*, 1996). Very high levels of fumonisin (>100 ppm in diet) may cause fatal pulmonary edema in pigs 1–4 hours after exposure (Marasas, 2001). Non-cardiogenic pulmonary edema has been associated with airway obstruction, cranial trauma, seizures or electric shock (Drobatz *et al.*, 1995). In horses, hyperhydration prior to exercise may be detrimental to respiratory function due to some degree of edema (Sosa *et al.*, 2002). Laboratory rodents receiving very high doses of paraquat may die with pulmonary edema and hemorrhage (Witschi and Last, 2001).

Some toxic agents, such as alloxan, which denude the alveolar capillary unit, reduce the likelihood of recovery (Witschi and Last, 2001), and may lead to significant collagen deposition (Pickrell and Villegas, research communication). If the changes are intense and architecture is severely compromised, pulmonary fibrosis may result. From some insults (histamine) an uneventful recovery may result. Several other insults (e.g., paraquat) cause sufficient injury to lead to healing by secondary intent (fibrosis).

We determine the extent of pulmonary edema by relating lung wet weight to body weight (Witschi and Last, 2001). Alternatively, lungs, lobes or slices can be weighed before and after drying. The accuracy of the second procedure is limited by the uniformity of the edema, but again this may be a useful indicator (Witschi and Last, 2001).

Acute respiratory distress, particularly acute pulmonary edema, is treated with diuretics such as furosemide, intranasal oxygen, bronchodilators, corticosteroids and alleviation of the underlying cause (Foreman, 1999). It is especially important to address the underlying cause in many of these conditions. Furosemide was formerly used in North America as a race-day preventive for exercise-induced pulmonary hemorrhage. The difficulty with using furosemide as a preventive is that furosemide may also be a performance-enhancing agent.

#### *Nasal and upper airway reactivity*

Upper airway irritant gases and particulates, especially ultra-fine particulates with greatly increased surface area, stimulate nasal (trigeminal) nerve endings (Witschi and Last, 2001). They also elicit avoidance reactions. Phosphine (PH<sub>3</sub>), for example, is liberated after ingesting zinc phosphide rodenticide; PH<sub>3</sub> irritates nasal and upper airway nerve endings resulting in avoidance reactions such as breath holding and subsequent paroxysmal breathing (Knight, 2006). Other agents, such as HCl, NH<sub>3</sub>, NO<sub>2</sub> and COCl<sub>2</sub>, appear to initiate serum leakage through toxic effects on epithelial cells or endothelial cells or both. Highly reactive molecules cause their reactions in a different manner. It is unlikely that ozone can penetrate fluid layers to cause direct toxicity; it is more likely that reactive molecules initiate a cascade of reactive oxygen substances such as aldehydes and hydroperoxides.

The lung contains most of the metabolizing P450 isoenzymes found in other tissues, but often at lower concentrations than in the rest of the body. These P450 isoenzymes are highly inducible. Activation of the enzymes is an initial defensive reaction which may contribute to healing and protecting the lung (Nel *et al.*, 2006). Although the P450 isoenzymes are clearly activated after exposure, it is not clear whether the response is sufficiently linear and consistent to use them as a biomarker of exposure. Other metabolizing enzymes in the lung include glutathione-S-transferase and glutathione peroxidase. In fact, the lung has been found to contain several forms of glutathione-S-transferase.

#### *Mediators of lung toxicosis*

Cytokines (tumor necrosis factor alpha (TNF $\alpha$ ), transforming growth factor  $\beta$  (TGF- $\beta$ ) and interleukin 1B (IL-1B)) are found in respiratory tract lavage fluid, and are associated with cultured whole lung tissue and of specific lung cells (lung epithelial cells and fibroblasts) (Witschi and Last, 2001). Cytokines can be used in studies to signal toxicant-induced lung damage. TNF $\alpha$  and IL-1B, for example, signal acute lung injury. TGF- $\beta$  and the cytokines signaling acute lung injury are part of the pathogenesis of pulmonary fibrosis. IL-1, IL-2, IL-5 and

IL-8 are thought to be essential components of epithelial lung cell injury. Finally, IL-4, IL-5 and IL-13 are thought to be associated with allergenic responses.

#### *Cell proliferation*

Toxicants can cause either reversible or irreversible lung changes (Witschi and Last, 2001). Toxic changes that cause major alterations in lung architecture usually cause irreversible lung injury. Severe tissue injury is followed by either tissue loss (emphysema) or healing by secondary intent (fibrosis). Although information about the typical responses associated with specific toxicants is available, it is still unclear how different responses are triggered.

When type I lung epithelial cells are injured and die following exposure to an oxidant gas, some type II lung epithelial cells proliferate, and begin to flatten and stretch to cover the denuded area. Clara cells proliferate following type II proliferation (Witschi and Last, 2001). Migration of blood monocytes to injured areas may trigger responses from other cells in the parenchymal lung unit, for example interstitial fibroblasts, myofibroblasts and endothelial cells. If the damage is relatively minor, the lung may heal with a relatively normal appearance, a higher total lung collagen content and minimal, if any, alteration of pulmonary function.

### **Chronic responses of the lung to injury**

#### *Fibrosis*

Healing by secondary intent, often called pulmonary fibrosis, may be either reversible or irreversible depending on the degree of injury. When lung injury is too severe, or persists too long to heal spontaneously, for example with high levels of paraquat, fibroblasts will attempt to fill the void left by type I lung epithelial cell death. Fibroblast proliferation may contribute to ventilation perfusion mismatch, making the lung less functional than when repaired by epithelial proliferation. In addition, there would be greater alteration of pulmonary architecture and increased numbers of interstitial fibroblasts. Increased fibroblasts making normal amounts of collagen would be expected to collectively make more collagen. If minimal to moderate alterations in pulmonary architecture are present the lung will spontaneously revert back to normal (Pickrell *et al.*, 1983). If greater alterations have been made to pulmonary architecture, the interstitial fibrosis may become irreversible and form dense fibrous scars (Pickrell *et al.*, 1983; Witschi and Last, 2001).

There are over 10 genetically distinct collagen types in animals and humans. Types I and III are major interstitial collagens in the lung. Type III collagen is more compliant

than type I. An increase in type I relative to type III collagen, which has been shown in experimental models of fibrosis caused by paraquat and asbestos, makes the lung even stiffer than if only an increased amount of collagen was present and the ratios of type I to type III collagen were unchanged (Witschi and Last, 2001).

### Emphysema

Emphysema has been defined as enlarged air spaces due to tissue destruction without fibrosis (Witschi and Last, 2001). The lungs become larger, more compliant and hyperinflated. Loss of gas exchange surfaces causes a reduction in gas exchange potential. Alveoli collapse, air spaces become distended and irregular, and air may become trapped. This makes it more difficult to expel air accumulated in distended alveoli. Patients become emaciated, have increasingly forced expirations and develop heave lines due to increasingly difficult and forceful expirations (Lowell, 1990).

Fibrosis is associated with local, heterogenic, compensatory emphysema. In areas where damage is sufficiently low, the lung heals normally. In areas where injury is more extensive, fibroblast proliferation and the excess production of collagen may result in fibrosis. In a still higher level of injury, even fibroblasts cannot effectively fill voids, healing by secondary intent fails and lung tissue is lost and emphysema forms. The extent to which this varied may be related to variations in local dose, the reserve of biological antioxidants or the balance between proteinase–antiproteinase (Pickrell *et al.*, 1983, 1987a, b).

### Asthma

In asthma, small conducting airways constrict at lower irritant concentrations than in normal lungs. Bronchoconstriction reduces airway diameter resulting in increased small airway airflow resistance. Symptoms include coughing, wheezing and progressive dyspnea (Witschi and Last, 2001).

Guinea pigs are highly sensitive to upper airway irritation and to subsequent development of bronchial asthma. Other animals that develop syndromes similar to human asthma include horses, dogs and cats (Padrid, 2000; Davis and Rush, 2002; McCue *et al.*, 2003; Pirie *et al.*, 2003; Reinero *et al.*, 2006). In horses, an asthmatic condition known as heaves is associated with inhaled endotoxins and organic dust particulates, which are thought to be synergistic in their effects (Pirie *et al.*, 2003). Potentially detrimental effects can be reduced by maintaining horses in “low dust” stables or at pasture, since these environments have significantly lower airborne dust and endotoxin levels (McGorum *et al.*, 1998). Poultry farmers have significantly higher incidences of chronic bronchitis. The major risk factor for respiratory symptoms was shown to be inadequate ventilation of

animal houses (Radon *et al.*, 2002). Airborne endotoxin can cause flu like symptoms in birds; this condition must be differentiated from infectious avian influenza (Joseph and Subbarao, 2005).

Specific allergen immunotherapy is a potential therapy for feline asthma. In one study, Rush Immunotherapy (RIT) blunted eosinophilic airway inflammation in experimental feline asthma. BAL cytokine profiles favoring a Th2 response developed after giving Bermuda grass antigen. RIT shifted the response to increased IFN- $\gamma$ , and IL-10 thereafter. The mechanism of RIT may involve changes in allergen-specific immunoglobulin production, induction of hyporesponsive lymphocytes or alteration of cytokine profiles (Reinero *et al.*, 2006).

### Lung cancer

In laboratory animals, spontaneously occurring malignant pulmonary tumors are rare, except for very old animals. If lung tumors do develop in laboratory rodents, they are generally peripheral adenomas instead of the more central tumors seen in humans. Adenomas usually originate from type II pulmonary epithelial cells, or Clara cells. These adenomas rarely have sufficient time to develop into carcinomas. Occasionally, lung tissue injury causes bronchogenic adenocarcinoma, which is an unusual lung tumor in cats; it may metastasize to the digits and to the abdominal wall. Average survival time for tumors metastasizing to the digits is only ~5 weeks (Van der Linde-Sippman, 2000; Petterino *et al.*, 2005).

Certain mouse strains (strain A and Swiss-Webster mice) with mutations in the K-ras genes similar to human carcinomas develop adenomas that increase in incidence after the inhalation of certain toxicants or carcinogens. These mice are potential models for human lung carcinogenesis (Witschi and Last, 2001).

Rat lungs occasionally contain lesions of epithelial cells surrounding material identified as keratin (Witschi and Last, 2001). The cells may compress lung parenchyma and occasionally invade it. These lesions are found more frequently in long-term tests of animals exposed to substances not considered to be carcinogens such as carbon black, titanium dioxide and certain man-made fibers. Controversy exists as to whether these are cysts filled with keratin, or can be classified as tumors.

In humans, it has been estimated that 80–90% of all lung tumors are caused by inhaling cigarette smoke (Witschi and Last, 2001). Dogs are companion animals that live with their owners over long periods of time. Although rare, some dogs developed either nasal or lung tumors after being exposed to owner's second-hand cigarette smoke. In described cases, the tumors were carcinomas – epithelial in origin and malignant. Dogs living for protracted periods of time in a home



with a smoker were more susceptible to lung cancer than those in homes of non-smokers (odds ratio = 1.6, 95% confidence interval 0.7–3.7). No relations were found between cancer rates and increasing the number of packs of cigarettes smoked per day or the proportion of time the dog spent within the home. However, dog breeds with short and medium length noses had an increased tendency to develop lung cancer (odds ratio = 2.4, 95% confidence interval 0.7–7.8). Dogs with long noses (dolicephalic breeds) tend to deposit carcinogenic particles in the nasal cavity, and may develop nasal carcinomas. Alternatively dogs with shorter noses (brachycephalic breeds) tend to allow carcinogenic particles to deposit in the upper airways and develop bronchogenic carcinomas (Reif *et al.*, 1992). The most frequent tumors in dog lungs, however, are mammary tumors that metastasize to the lung.

## Agents known to produce lung injury in animals

### Zinc phosphide

Poultry are highly susceptible to zinc phosphide poisoning. For example, approximately 200 chickens inadvertently given zinc phosphide were found dead with no clinical signs after the flooring of a slat-and-litter house was breached (Tiwary *et al.*, 2005). Gross necropsy revealed intense congestion of the viscera; histopathologic examination revealed severe pulmonary edema and congestion of the chickens' lungs, hearts, livers and kidneys (Tiwary *et al.*, 2005).

### Smoke

Smoke inhalation injury is common in animals when confinement does not allow them to move away from smoke-filled air. Cardiac dysfunction in sheep following combined burn and smoke injury was mostly related to hypovolemia, and was improved by aggressive fluid therapy. However, later myocardial contractile dysfunction seemed to be correlated with smoke inhalation injury (Soejima *et al.*, 2001). In chickens, the interparabronchial septal spaces were measurably thickened and engorged as a result of hydrostatic pulmonary edema, similar to the effect of hydrostatic pulmonary edema in mammals (Weidner *et al.*, 2002). Under similar conditions, air sacs thickened, and microvilli had increased density (Weidner, 2000).

### Overheated frying pans

Exposure of caged birds to gases released from overheated non-stick cookware has been shown to result in a high percentage of deaths (Brown *et al.*, 1997). Most of

these birds have pulmonary edema and hemorrhages. Proximity to the source is an important risk factor. Birds in adjacent rooms may have only minimal pulmonary reactions compared to birds close to the source, even when the total exposure is similar for both (Brown *et al.*, 1997). The mechanisms of these differences are not known at this time. It has been suggested that physical-chemical changes occur in released substances over time, making them less toxic. Alternatively, particles released from overheated surfaces in the same room are smaller in diameter, and more numerous, closer to the source, whereas those further away tend to coalesce into larger particles. Thus, birds in the same room would be exposed to more surface areas per unit mass than those in adjacent rooms.

### Tryptophan and *Perilla frutescens*

Tryptophan toxicity is associated with cattle consuming tryptophan in lush green forages (Pickrell and Oehme, 2004). The rumen converts tryptophan to 3-methyl indole (MI), which is metabolically activated by cytochrome P450 to a reactive compound in the lung. In a similar toxicosis, perilla ketone and 4-ipomeanol from *Perilla frutescens* (beef steak plant) damage endothelial cells and type I pulmonary epithelium (Nicholson, 2004; Pickrell and Oehme, 2004).

At high doses, MI leads to pulmonary edema, excess proteolysis, tissue destruction, emphysema and death within 6–24 hours after the onset of clinical signs (Pickrell and Oehme, 2004). Several pounds of green forage containing 4-ipomeanol may be fatal to a cow within 1–2 days (Nicholson, 2004). At lower exposure levels, animals may have respiratory signs (depression, respiratory grunt, wheeze and froth flowing out of the nostrils), but will recover over 24–72 hours. These animals will recover in 24–72 hours with minimal to no physiological impairment. However, proliferation of type II alveolar cells may persist with no demonstrable physiological consequences. If exercised, cattle with intermediate signs can be pushed into the acute syndrome; they will worsen rapidly, develop significant lung edema and may die (Pickrell and Oehme, 2004).

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# Cardiovascular toxicity

Csaba K. Zoltani

## INTRODUCTION

Cardiotoxicity is a dysfunction of the electrophysiology of the heart. Primarily this manifests itself in inadequate oxygenation of the blood, affecting all organ functions. The electrophysiological dysfunction in animals is primarily caused by phytotoxins, chemical toxicants and venoms.

The ingested poisonous feedstock and environmental toxins may lead to a wide variety of symptoms. In some cases, cardiotoxicity is only ancillary for the initial diagnosis but its recognition and subsequent treatment may be of primary importance for the outcome.

Cardiotoxicity is evidenced by changes in the electrocardiogram (ECG). Initial indicators of cardiac injury include increases in the amplitude of the T-wave and ST-segment. In addition, biomarkers of cardiac toxicity give insight into the nature of the injury. These include the levels in the serum of the two isoforms of cardiac troponin I and T, whose levels rise after ischemia and indicate myocardial necrosis. Also myoglobin, which is released by injured myocardial cells, is present. Levels of creatine kinase (CK), particularly CK-MB, rise after the onset of infarction or even after tachycardia, and are indicative of cardiac muscle damage. Lactic dehydrogenase (LDH), which catalyzes the conversion of lactate↔pyruvate, is present in many tissues with variable activities of its five isoenzymes activities (LDH-1→LDH-5). LDH-1 isoenzyme is found primarily in heart tissue, while LDH-2, LDH-3, LDH-4 and

LDH-5 are found in many other tissues. Tissue damage elevates LDH activity. When LDH-1 > LDH-2, this indicates heart attack or cardiac muscle injury. FABP3, an intracellular fatty acid-binding protein and a marker for myocardial damage, is also released into plasma after myocardial injury.

This chapter addresses the primary cardiac problems encountered in animals as a result of unsuitable feedstock, or the introduction of poisons into their system by alternate means.

## PLANT-RELATED CARDIOTOXICITY

The primary cause of cardiotoxicity in animals is the ingestion of toxic plants. Most plants are widely distributed, though possibly geographically contained, and toxicity is dependent on the season, environmental conditions, particular ingredient and part of the plant accessed. Excellent references and databases on plants exist that give detailed information (Kingsbury, 1964; Frohne and Pfander, 2005; Burrows and Tyrrel, 2006; Panter *et al.*, 2007; FDA, 2008). Additional information on plants and their effect on the cardiovascular system is described elsewhere (Beasley, 1999; Knight and Walter, 2001).

Plants can contain a variety of toxic chemicals and the majority of them are either glycosides or alkaloids that produce cardiac toxicity.



## GLYCOSIDE-CONTAINING PLANTS (SEE TABLE 16.1)

### *Digitalis purpurea* (foxglove)

Important cardiac drugs are derived from foxglove, including digitalis that helps to increase the force of contraction of the heart muscle. However, the effect of cardiac glycosides is dose dependent and has a very narrow therapeutic range, i.e., slight overdosage induces toxicity. Glycosides, with a digitalis-like effect, are lethal in domestic animals upon ingestion of 100–200 mg/kg body weight. The main toxic glycosides in foxglove include digitoxin, digitonin and digitalin. The LD<sub>50</sub> of digitoxin in cats is 0.18 mg/kg body weight, and in guinea pigs is 60 mg/kg body weight. Poultry as well as ruminants are also affected (Thomas *et al.*, 1987).

In general, the mechanism of toxicity of digitalis glycosides at the cellular level involves interference with the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump, resulting in decreased intracellular potassium (K<sup>+</sup>) and consequently decreased resting potential. At high doses, glycosides in the heart affect an electrical conduction, resulting in an increased vagal tone to the atrioventricular (AV) and the sinoatrial (SA) nodes. This may culminate in cardiac arrhythmias and asystole (cardiac arrest). Additionally, they cause myocarditis, myocardial lesions and hemorrhage.

Digoxin overdose induces ECG changes, such as ST-depression, T-wave inversion, PR-interval prolongation and QT-interval decrease, thereby leading to the signs of cardiac toxicity. Digoxin interferes with the binding sites of potassium ions of the Na<sup>+</sup>/K<sup>+</sup>-ATPase

pump. This increases the level of Na<sup>+</sup> ions in the myocytes that causes higher intercellular calcium ions resulting in increased cardiac contractions. In addition, cardiac glycosides have vagotonic effects, resulting in bradycardia and cardiac arrest (Beasley, 1999; Liu *et al.*, 2010).

Cardiac symptoms caused by glycosides of *Digitalis purpurea* include strong cardiac contractions, prolonged diastole, pulse and cardiac rhythm abnormalities, hyperkalemia and ventricular tachycardia (Lin *et al.*, 2010).

Treatment for digitalis poisoning is usually symptomatic and involves multi-dose activated charcoal and atropine.

### *Asclepias* spp. (milkweeds)

Milkweed is a perennial plant indigenous to the Great Plains and from North Carolina to Maine (Kingsbury, 1964; Stevens, 2000). The most toxic is labriform milkweed, followed by the western whorled milkweed. Milkweeds exude a milky juice from their broken surfaces. Most milkweeds contain a mixture of steroidal glycosidic cardenolides that are toxic to animals (Panter *et al.*, 2007). The cardenolides induce cardiotoxicity by inhibiting Na<sup>+</sup>/K<sup>+</sup>-ATPase activity.

Horses, cattle, sheep, goats and poultry are all susceptible, with a dosage of whorled milkweed of 0.1–0.5% body weight of the animal being fatal. Clinical signs appear within few hours of plant ingestion. Cardiac signs include bradycardia or tachycardia, hypotension and arrhythmia. Death occurs within a few days of plant exposure. Histological lesions and myocarditis are

TABLE 16.1 Glycoside-containing plants

Botanical name	Common designation	Cardiac symptoms	Ancillary cardiac effect	Toxicity
<i>Asclepias</i> spp.	Milkweed	weak, rapid pulse, arrhythmia	hypotension, arrhythmia	dosage of 0.5% b.w. fatal for horses, cattle
<i>Persea Americana</i>	Avocado	heart failure, cardiomyopathy	necrosis of myocardial tissue, inflammation of the heart; horses, rabbits, goats, ostriches affected	sheep fed 2.5 g/kg b.w. for 32 days developed cardiac insufficiency
<i>Digitalis purpurea</i>	Foxglove	cardiac rhythm abnormality, hyperkalemia	stronger cardiac contractions and slower contractions through stimulation of the vagus, prolonging diastole; affects cats, dogs, livestock	LD <sub>50</sub> (cats) 0.18 mg/kg b.w., guinea pigs 60 mg/kg b.w.
<i>Nerium</i> spp.	Oleander	bradycardia and/or tachycardia, ventricular fibrillation	in sheep depression of ST on ECG, hyperkalemia, serum potassium may increase two-fold, 2nd degree AV block	LD <sub>50</sub> (goats) 330 mg/kg, b.w., in sheep 110 mg/kg b.w.
<i>Convallaria majalis</i>	Lily of the valley	sinus tachycardia, heartblock	cardiac signs may be delayed, heart becomes hyperirritable	LD <sub>50</sub> (cat) 0.14 mg/kg b.w.
<i>Rhododendron</i> spp.	Azalea, rhododendron	arrhythmia, hypotension, weak heart rate	contains the toxin grayanotoxin	LD <sub>50</sub> (mice) 5.1 mg/kg b.w.
<i>Kalmia</i> spp.	Laurel	bradycardia	excitable cells remain depolarized	LD <sub>50</sub> (rats) 2–5 mg/kg b.w.

common (Clark, 1979). Treatment includes lavage with activated charcoal and a cathartic.

### *Persea americana* (avocado)

*Persea americana* of the family *Lauraceae* is a tree found in Mexico and commercially grown in the United States (mainly in California and Florida). The leaves, fruit and seeds of the plant are considered to be toxic, with the leaves being the most toxic. A variety of animal species have been naturally or experimentally intoxicated by *P. americana* including cattle, goats, horses, rats, mice, rabbits, sheep, ostriches, dogs and pet birds. The toxic principle is persin, which causes myocardial necrosis in birds and mammals. Persin also targets mammary glands in lactating animals. Sheep fed 2.5g/kg body weight for 32 days developed cardiac insufficiency. Upon ingestion of avocado, dogs also develop myocardial damage. At high doses, exceeding 100mg/kg body weight, the myocardium is affected with necrosis of myocardial fibers (Oelrichs *et al.*, 1995). Goats fed fresh avocado showed cardiac distress (Grant *et al.*, 1991). Recently, Poppenga *et al.* (2010) reported acute avocado poisoning in goats. Gross and microscopic findings included pulmonary congestion, and multifocal, necrotizing, neutrophilic myocarditis. Treatment is symptomatic and nonspecific.

### *Nerium* spp. (oleander)

*Nerium oleander* (oleander) is an evergreen shrub of the *Apocynaceae* family that thrives principally in subtropical regions. All oleanders contain cardenolides that exert positive inotropic effect on cardiac muscle. Their action is derived from inhibition of the plasmalemma (cell membrane)  $\text{Na}^+/\text{K}^+$ -ATPase.

In sheep, a dose of 110mg/kg body weight led to death preceded by decreased heart rate, tachyarrhythmia and conduction defects affecting sinus node, the AV node and elevated CK (Eddleston *et al.*, 2000). ECG revealed bradycardia, AV block, depression of the ST segment, tachycardia and ventricular fibrillation (Aslani *et al.*, 2004). Histopathological examination revealed myocardial degeneration. In goats, ECG showed second degree AV block (Barbosa *et al.*, 2008). Antidigoxin Fab treatment restores sinus rhythm and is the only proven therapy for yellow oleander poisoning (Rajapakse, 2009).

### *Convallaria majalis* (lily of the valley)

Lily of the valley is an herbaceous perennial woodland plant native to northern climates. Cardenolides concentrations are highest in the roots, but all plant parts are

of concern. There are over 30 glycosides and convallatoxin is the most toxic with an  $\text{LD}_{50}$  of 0.08mg/kg body weight (Fenton, 2002). Convallatoxin produces cardiac toxicity by inhibiting the enzyme  $\text{Na}^+/\text{K}^+$ -ATPase. Overdose causes intoxication, sinus tachycardia, heart block and fibrillation, culminating in cardiac arrest. Cats are extremely sensitive to lilies, with the ingestion of two leaves resulting in death (Fitzgerald, 2010). Fluid diuresis, before the onset of renal failure, has been used as treatment.

### *Rhododendron* spp. (azalea, rhododendron)

Rhododendron is a genus of over 600 species that includes azalea. Its toxic principle is grayanotoxin (Aker *et al.*, 1976), a compound that inhibits  $\text{Na}^+/\text{K}^+$ -ATPase. The grayanotoxin binds to the muscarinic receptors. Bradycardia results from the peripheral vagal stimulation. Severe cases lead to cardiovascular collapse. The toxic principle interferes with cardiac muscle and clinical effects are apparent several hours post-ingestion (Sharma *et al.*, 2009). Cardiovascular symptoms include hypotension, cardiac arrhythmias and weak heart rate. The severity of toxicity also depends on the route of exposure. The  $\text{LD}_{50}$  in mice is 5.1mg/kg body weight (Barceloux, 2008). Treatment is symptomatic.

### *Kalmia* spp. (laurel, lambkill)

Laurel is an evergreen shrub of the family *Ericaceae*, native to North America. The plant contains the glycoside grayanotoxin which is toxic to ruminants. The  $\text{LD}_{50}$  in rats is 2–5mg/kg body weight (Frohne and Pfander, 2005). Minimum toxic dose in cattle is 0.4% body weight (Beasley, 1999).

A grayanotoxin, also known as acetylandromedol, binds to the sodium ion channel receptors involved with activation and inactivation of the cells. With inactivation blocked, the excitable cells remain depolarized. Cardiac symptoms include low blood pressure, bradycardia and also ventricular tachycardia.

Therapy includes atropine and also vasopressors to raise the blood pressure. Arrhythmias produced by grayanotoxin I can be reversed by tetrodotoxin (Aker *et al.*, 1976; Ku *et al.*, 1977).

## CYANIDE-CONTAINING PLANTS

Approximately 2500 plants contain cyanogenic glycosides (Vetter, 2000). Hydrocyanic acid/prussic acid is

commonly formed in the seeds and leaves of sorghum plants and also from cassava. It is a rapidly acting poison that inhibits the action of enzymes (cytochrome oxidase) that links oxygen with the erythrocytes. Plants with only 20mg of cyanogenic glycosides per 100g (Allison and Baker, 2007) can kill livestock by asphyxia. By inhibiting cytochrome oxidase, cyanide induces cellular hypoxia. Since oxygen cannot be utilized, ATP is no longer formed. Cardiac symptoms include tachycardia, dysarrhythmia and petechial hemorrhage of the heart and other organs. ECG changes include elevation or depression and shortened ST-segment, fusion of the T-wave and QRS segment (Holstege *et al.*, 2010). The amount of hydrocyanic acid formed by a plant varies, but the lethal dose for a mouse is 3.7mg/kg body weight and for cattle and sheep it is 2.0mg/kg body weight (Speijers, 2003). Plants containing cyanogenic glycosides in excess of 20mg/100g of plant material are avoided in feedstock.

Ruminants are more likely to be poisoned than monogastric animals (horses and pigs) because the lower pH in the stomach of monogastric animals destroys enzymes that convert cyanogenic glycosides to hydrocyanic acid.

Antidotes include sodium nitrite and sodium thiosulfate.

ALKALOID-CONTAINING PLANTS  
(SEE TABLE 16.2)

The alkaloids from plants of roughly 20 families are known to exert cardiac toxicity. These alkaloids are the

polycyclic diterpenes that act as neuromuscular blocking agents. There are two classes of steroidal alkaloids; solanum and veratridine (Schep *et al.*, 2006). The solanine alkaloid opens the potassium channels of mitochondria and increases Ca<sup>++</sup>, while tropane alkaloids are anticholinergic and cause arrhythmias, bradycardia and tachycardia. Common plants that contain toxic alkaloid(s) include Larkspur, yew, monkshood, ground hemlock and death camas. The lily family contains the alkaloid veratridine that causes persistent opening of voltage-gated sodium channel and significantly reduces channel conductance.

*Delphinium* spp. (larkspurs)

Larkspurs consist of 250 species of annual and biennial flowering plants of the buttercup family *Ranunculaceae*, distributed throughout the Northern Hemisphere. Their ingestion has been responsible for extensive losses in cattle (Pfister *et al.*, 1999; Green *et al.*, 2009a; Green *et al.*, 2009b). The plant contains numerous norditerpenoid alkaloids and their toxicity greatly varies (Panter *et al.*, 2002; Welch *et al.*, 2008). Three alkaloids are of major concern: methyllycacontine (MLA), 14-deacetylnudicauline (DAN) and nudicauline (NUD). The LD<sub>50</sub> is 25–40mg/kg body weight, depending on the type of alkaloid present.

Intoxication occurs when the nicotinic acetylcholine receptors are blocked. There are species differences in the symptoms, but the usual cardiac signs include rapid and irregular heartbeat and hypotension. The effect of larkspurs alkaloids are counterbalanced by the anticholinesterase agent, such as neostigmine or physostigmine. Anticholinesterases alleviate the clinical symptoms

TABLE 16.2 Alkaloid-containing plants

Botanical name	Common designation	Cardiac symptoms	Ancillary cardiac effect	Toxicity
<i>Delphinium</i> spp.	Larkspur	irregular tachycardia	methyllycaconitine is antagonistic of nicotinic receptors; important role in poisoning of livestock	LD50 (cattle) 25–40mg/kg b.w.
<i>Aconitum</i>	Monkshood	hypotension, myocardial depression, conduction disturbance, tachycardia	contains methyllycaconitine acts as NM blocking agent	LD50 (mice) 130–280mg/kg b.w.
<i>Taxus</i> spp.	Yew	bradycardia, acute cardiac failure	depresses conduction of depolarization through the heart, no significant lesions	LD50 (rabbit) 8mg/kg b.w., LD50 (mice) 15mg/kg b.w.
<i>Zygadenus</i> spp.	Death camas	weak, irregular pulse, decrease in blood pressure due to dilation of arterioles	constricting veins, no specific lesions on autopsy, sheep especially vulnerable	sheep lethal dose: 0.6–6% b.w.
<i>Eupatorium rugosum</i>	White snakeroot	congestive heart failure	cardiac muscle degeneration	0.5–2.0% b.w. consumption intoxicates
<i>Astragalus</i>	Locoweed	congestive right-heart failure	accumulates selenium, heart affected	toxin content greater than 0.001% causes poisoning

in cattle (Green *et al.*, 2009a). Physostigmine given IV at the rate of 0.08 mg/kg body weight has been used under field conditions to relieve symptoms, and may need to be repeated.

### ***Aconitum* (monkshood)**

Monkshood is a herbaceous perennial found in elevated regions of the Northern Hemisphere. All parts of the plant are poisonous and contain polycyclic diterpene alkaloids mainly of the aconitine type. These alkaloids have affinity for the open state of the voltage-sensitive  $\text{Na}^+$  channel making them refractory to excitation (Chan, 2009). The resting  $\text{Na}^+$  permeability of excitable cells is increased, resulting in incomplete inactivation of  $\text{Na}^+$  channels in nerves (persistent activation) and a decrease in ion selectivity. This causes depolarization of resting nerve and muscle and an increase in repetitive discharge. With low dosage there is stimulation of medullary vagal centers, causing decreased heart rate and blood pressure. Higher dosage of aconitine predisposes to a variety of arrhythmias, including atrial flutter and fibrillation, due in part apparently to the complex effects of aconitine on the CNS, which in turn affect the adrenergic and cholinergic systems (Burrows and Tyrl, 2001). The  $\text{LD}_{50}$  in mice for aconitine is 130–280 mg/kg body weight, depending on the plant. Aconitine causes hypotension, bradycardia, ventricular tachycardia and conduction disturbances. Treatment is symptomatic and supportive.

### ***Taxus* spp. (yew)**

The yew is an evergreen shrub found throughout North America. Ingestion of clippings from the plant causes poisoning in ruminants as well as in monogastrics. The toxic principles, taxine A and B alkaloids, cause an increase in the cytoplasmic  $\text{Ca}^{2+}$  and interfere with  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ion channel conductance that precedes bradycardia and diastolic cardiac arrest. Taxine B induces increased AV conduction times, longer QRS and an absence of P-waves on ECG (Tiworthy *et al.*, 2005).

In ruminants, 0.5% of body weight, and in monogastrics (horses), 0.1% of body weight, ingestion is enough to cause clinical signs of toxicity. In mice, the  $\text{LD}_{50}$  is 15 mg/kg body weight and cardiac symptoms include arrhythmias, possibly culminating in cardiac failure (Wilson *et al.*, 2001).

Observed blockade of  $\text{Na}^+$  or  $\text{Ca}^{2+}$  channels also causes the Brugada-like syndrome (Veltmann *et al.*, 2009). No specific treatment is known for yew poisoning except atropine and lidocaine for symptoms and treatment directed toward stabilization of cardiovascular function.

### ***Zigadenus venenosus* (death camas)**

Death camas is an abundant perennial plant found in western states. It grows early in the spring but enters dormancy when soil moisture declines. It is poisonous to livestock upon ingestion, with death occurring within a few hours. The toxic principle is the cevanine steroidal alkaloid, i.e., zigacine (Beasley, 1999). In sheep 0.6–6% of body weight ingestion is fatal. Zigacine causes weak, fast, irregular pulse, bradycardia, drop in blood pressure, necrosis of cardiac muscle and cardiovascular failure. There is no known treatment for death camas poisoning.

### ***Cicuta maculata* (water hemlock)**

Water hemlock is one of the most toxic plants found in North America (Panter *et al.*, 1988). Water hemlock is a biennial and tall plant and grows in wet surroundings. The toxic principle is cicutoxin which affects the central nervous system. It acts on the GABA (gamma-aminobutyric acid) receptor, blocking the  $\text{Cl}^-$  channel that causes neuronal depolarization and seizures. Death occurs due to respiratory failure. One g/kg body weight will kill sheep and 230 g horses and cattle. Due to its severe toxicity in cattle, it is also called “cowbane.”

Cardiovascular effects include alterations in blood pressure, heart rate, a widening of the PR interval on the ECG, supraventricular tachycardia and ventricular fibrillation (Schep *et al.*, 2009). Degeneration and fibrosis in the heart is observed and the blood serum shows increases in AST and LDH, enzymes which are released upon injury to the heart or liver.

Treatment, though difficult and usually not in time due to the rapidity of the poisoning, includes intravenous administration of barbiturates to control seizures.

### ***Eupatorium rugosum* (white snakeroot)**

White snakeroot is a member of the *Asteraceae* family native to the eastern United States. An erect plant is up to 1.5 meters tall with the leaves resembling those of the nettle. Consumption of plants at 0.5–2.0% of body weight causes poisoning in livestock (Doyle *et al.*, 1949). The plants are poisonous to horses, goats and sheep. White snakeroot contains the toxin tremetol. Tremetol passes through the milk in cows, and thus contaminated milk causes sickness in suckling calves and humans. It is important to mention that tremetol is cumulative in that repeated exposure leads to intoxication.

The onset of clinical signs begins 2 to 3 weeks post-ingestion. In horses it causes congestive heart failure (Maratea, 2003). The enzymes CK, ALP (alkaline



phosphatase) and AST are elevated and cardiac muscle degeneration occurs. Pale linear streaks in the myocardium, as well as fluid in the pericardiac sac, have been observed. In horses, swelling in the neck area is also noted. Rapid heart rate, elevation of ST and variable QRS complexes in the ECG and cardiac arrhythmias may be present. Treatment consists of supportive care.

### *Astragalus* (locoweed)

Locoweed is found throughout the western United States and Canada. This perennial plant contains the toxic principle indolizidine alkaloid swainsonine, and is toxic to livestock. A toxic content of locoweed greater than 0.001% can cause poisoning. Cytoplasmic vacuolation of cells of the central nervous system occurs. It increases the severity of "high mountain disease," a hypoxia-induced pulmonary hypertension and hypertrophy of the right ventricle of animals living at elevated altitudes. Sheep, cattle and horses are affected. Some of the *Astragalus* species also accumulate large amounts of selenium, complicating the effect on the heart.

Pathological effects include neurological damage and congestive right-heart failure. The toxin inhibits the lysosomal enzyme  $\alpha$ -mannosidase. Treatment includes fluid therapy with potassium supplementation, dopamine and diazepam.

## OXALATES

Many oxalate-containing plants, such as philodendron, are kept for their ornamental beauty and are accessible to pets. If ingested in large quantities, a significant amount of oxalates can be absorbed into the bloodstream. Oxalic acid combines with calcium and forms the crystals of calcium oxalates. Although these crystals are primarily deposited in the kidneys, they can also be deposited in other organs, including the heart. In humans, oxalosis is known to cause cardiac abnormalities, including heart block (Coltart and Hudson, 1971), which can be linked to decline in renal function (Mookadam *et al.*, 2010). Cardiac manifestation of oxalosis has not been actively pursued in animals, but some data are available. Ingestion of sodium oxalate results in weak and irregular pulse, hypotension and ultimately cardiovascular collapse (VonBurg, 1994). Sheep and cattle grazing on *Rumex crispus* (curled dock) native to Europe and Western Asia may suffer with tetany, recumbency and death.

The formation of calcium oxalates also leads to hypocalcemia which interferes with electrical activity of the

heart as it inhibits the calcium pump active in the generation of the action potential and eventually results in the relaxation of the muscles. The reduced activity of the heart can culminate in cardiac arrest.

Therapeutic measures include induction of vomiting, and oral dosing of calcium lactate to reduce further absorption of oxalates.

## GOUSIEKTE

The Rubiaceae is the fifth largest flowering plant family spread worldwide but is found mostly in the warmer climates. An economically important member of the family is *Coffea arabica*, the source of coffee beans. Gardenia (*Gardenia jasminoides*) is another plant of the frost-free zones planted worldwide.

Four to 6 weeks after ingestion of plants of the Rubiaceae family, ruminants, without forewarning, drop dead with heart failure (Kellerman, 2005). Called gousiekte (Afrikaans name meaning quick sickness), the disease affects sheep and cattle and is characterized by myocardial necrosis consisting of a loss of myofibrils, cardiac dilations and replacement of myocytes with collagenous tissue. In the terminal phase of the disease, the heart has a rounded, flabby appearance without an apex and has attenuated papillary muscles. In the subendocardial region of the apex and the left ventricular wall, the principal lesions are degeneration of myofibers.

The toxic principle pavetamine from *Pavetta harborii* inhibits myocardial protein synthesis without affecting myocardial fibers or other organs. The disease is characterized by cellular injury and is not a specific heart disease. It terminates in dilated cardiomyopathy and congestive heart failure (Hay *et al.*, 2008; Prozesky, 2008). Sheep dosed daily at 25mg/kg body weight, after 4 weeks, showed typical signs of cardiac failure with galloping heart rhythm, decrease of ejection fraction of the left ventricle (from 60 to 30%) and the pumping efficiency of the left ventricle with respect to the right ventricle decreased (van der Walt and van Rooyen, 1977). Goats fed pavetamine showed ECG changes and tachycardia. The electrophysiology of the pacemaker cells was affected. In sheep, T-wave inversion was common as was hypokalemia.

## MUSHROOMS

Some of the mushrooms are toxic and often get mixed into animal feed or ingested by pets. The toxins present

in the mushrooms are amatoxins (cyclic octapeptides) (Berger and Guss, 2005). Amatoxins inactivate RNA polymerase II in cell nuclei thereby preventing protein synthesis, and curtailing cell metabolism (Duffy, 2008; Chang, 2009).

Amatoxins cause damage to cardiac tissue, as evidenced by hemorrhage, cloudy swelling and degeneration. Elevations of cardiac enzymes (Unverir *et al.*, 2007), including CK-MB isoenzyme and myoglobin, are noted. Heart block and bradycardia have been observed. Also, sinus tachycardia and hypotension are typical clinical cardiac symptoms.

In dogs, electrolyte abnormalities and bradyarrhythmia (SA exit block and sinus arrest) symptoms reminiscent of muscarinic intoxication were noted and relieved by atropine (Lee *et al.*, 2009). Treatment aims at minimizing amatoxins absorption by administering activated charcoal and hydration maintenance.

## XENOBIOTICS THAT MAY CAUSE CARDIAC TOXICITY

### Arsenic

Although much native vegetation contains arsenic, the sources of intoxication usually are sprays or ingestion of feed contaminated by arsenic (Selby *et al.*, 1977). Arsenic in drinking water also poses an additional risk of cardiovascular effects including hypertension, prolongation of the QT portion of the ECG, indicating disorder of the heart's conduction system and favoring cardiac arrhythmia (Mordukhovich *et al.*, 2009; el Bahri and Ben Romdane, 1991).

Large doses of arsenic lower blood pressure and slow the pulse. In frogs the heart stops in diastole due to the paralysis of the motor ganglia. Since it is quick acting, cardiovascular collapse is to be expected. Treatment includes gastric lavage and intravenous administration of sodium thiosulfate.

### Ionophores

Ionophores are carboxylic polyether antibiotics that are given as feed additives to cattle. Ionophores alter the flow of cations across cell membranes and reduce Gram-positive bacteria responsible for bloat. Since ionophores have a narrow range of safety, feed mixing errors facilitate poisoning. In horses this leads to cardiac muscle damage and in cattle both skeletal and cardiac muscle damage.

The cardiac signature of the poisoning in horses manifests by prolongation of atrial and ventricular

depolarization and repolarization (Hall, 2006). Also, depression of the ST segment, absence of the P-wave, ventricular tachycardia, AV block and atrial fibrillation are noted. Cardiac lesions and heart failure occur. In cattle, QT and QRS prolongation, first degree heart block and T-wave amplitude increases are typical. In cattle also, cardiac lesions and heart failure are possible. Sheep also have cardiac muscle damage. The LD<sub>50</sub> of monensin is 2–3 mg/kg body weight in horses, 20–34 mg/kg body weight in cattle and 10–12 mg/kg body weight in sheep and goats (Doonan *et al.*, 1989).

Besides the growth-promoting function of ionophores in ruminants, it is used as a coccidiostat in poultry. Cardiac effects of infested poultry include reduced lipid levels, reduced heart weight, low heart rates and low blood pressure. Monensin has a narrow therapeutic window and intoxication causes muscle necrosis and myoglobinuria. Targets include cardiac muscles. It causes inotropism, tachycardia, cardiomyopathy and cardiac failure.

There is no antidote available for monensin poisoning.

### Fertilizers

Accidental ingestion of fertilizer can cause serious poisoning in animals (Villar *et al.*, 2003). Predominantly, though not exclusively, nitrates cause the damage. Rabbits exposed to ammonium sulfate fertilizer suffered cardiac arrest (Sato *et al.*, 1999). In another case with fertilizer containing nitrogen, phosphoric acid and potassium, the rabbits suffered hyperkalemia, hyperammonemia and metabolic acidosis that expressed itself on the cardiac system as ventricular tachycardia translating into ventricular fibrillation and death. However, no alterations were noted on histopathological examination of the heart. There is no specific antidote for fertilizer poisoning.

### Herbicides

There are a large number of herbicides, characterized by differing chemical structures, that cause various effects on the heart (Gupta, 2007). The ingestion of herbicide-treated plants can induce electrolyte abnormalities and low blood pressure.

Paraquat is a dipyridyl herbicide, which generates excess reactive oxygen species (Ge *et al.*, 2010). In rats, paraquat caused severe heart damage, including hemorrhage in the myocardium and alterations in ECG (Noguchi *et al.*, 1990). It also elicited sinus tachycardia but no arrhythmia.

An effective antidote is not available. Bentonite or fuller's earth can be given orally to detoxify bipyridyls.

## Pesticides

### Organophosphates and carbamates

Organophosphate (OP) and carbamate (CM) pesticides produce toxicity by inhibiting acetylcholinesterase (AChE) activity. Inactivation of AChE results in excessive ACh accumulation at the synapses and at neuromuscular junctions leading to an overstimulation of the muscarinic and nicotinic ACh receptors.

Classical cardiac signs of OP poisoning include prolonged QT interval, ST-T changes, sinus tachycardia or bradycardia and hypertension or hypotension (Saadeh *et al.*, 1997; Abraham *et al.*, 2001; Karki *et al.*, 2004).

The most effective antidote for OP and CM poisoning is the muscarinic receptor antagonist atropine sulfate. Against OPs, oximes are used to reactivate the inhibited AChE (Boelsterli, 2007; Kose *et al.*, 2009).

### Amitraz

Amitraz is a triazapentadiene compound used in flea collars of dogs. As an insecticide and acaricide, it exerts toxicity by inhibiting the enzyme monoamine oxidase (MAO) and works as an  $\alpha_2$ -adrenergic receptor agonist. The cardiac effects of  $\alpha_2$ -adrenergic agonists include bradycardia, first and second degree atrioventricular blockage and diminished cardiac output (Malmasi and Ghaffari, 2010). ECGs performed on an English bulldog poisoned with amitraz showed QT elongation, a precursor to ventricular arrhythmia. Amitraz apparently lengthened the QT interval by reduction of the heart rate. Thermogenesis was also affected. Hypothermia decreased the depolarization of the pacemaker cells, so that bradycardia ensued.

## ANCILLARY CAUSES OF CARDIAC TOXICITY

## Arthropods

### Scorpions and spiders

Envenomation by arthropods (scorpions and spiders) is a serious health concern to humans and animals in many countries. Scorpion venoms can exert peripheral as well as central effects (Gwaltney-Brant 2011). In general, severe envenomation by scorpions produces the signs of cardiac and respiratory rhythm, arterial hypertension that can progress to congestive heart failure, pulmonary edema and shock. There can be frequent vomiting, diarrhea, salivation and alternating restlessness with somnolence and sometimes convulsions. Effects of scorpion venom are more pronounced on the cardiac system

(Murthy *et al.*, 1999; Dorce *et al.*, 2009). In dogs and rabbits, 3.5mg/kg body weight of scorpion venom causes an increase in LDH and CK-MB enzyme levels. ECG changes in T-waves (biphasic) and ST-segment and electrical alterations indicate myocardial damage, ancillary arrhythmias, conduction defects and infarction-like patterns (Gueron *et al.*, 1992; Bentur *et al.*, 2003). Scorpion toxin includes potassium channel blocking peptides (Kumar *et al.*, 2011). The venom of the death stalker scorpion (*Leiurus quinquestriatus*) includes chlorotoxin, which blocks small conductance of chloride channels.

Treatment for a scorpion sting is symptomatic. Hypertensive symptoms are treated with vasodilators. Scorpion antivenoms are available.

### Bees, wasps, hornets, yellow jackets and ants

The venom of bees, wasps, hornets, yellow jackets and ants is poisonous. A lethal dose is approximately 20 stings/kg body weight in most mammals (Fitzgerald and Flood, 2006). The severity of the stings is increased by anaphylactic reactions.

Stings can cause myocardial infarction and myocarditis. Increases in serum enzyme levels and necrosis in the myocardium have been reported (Levine, 1976; Ferreira *et al.*, 1995). Mast cells in cardiac tissue may be affected by their mediators which have cardiotoxic effects, including arrhythmogenesis. Wasp stings can cause Mobitz Type I heart block and myocardial infarction (Wagdi *et al.*, 1994). ECG changes, myocardial necrosis and arrest of cardiac beats were noted after envenomation by Africanized honey bee venom in Wistar rats. Honeybee sting can produce significant elevations of the ST-segment, CK-MB activity and troponin I and T levels.

Treatment includes antihistamines, steroids, bronchodilators and  $\alpha$  and  $\beta$ -receptor agonists.

### Snakes

Six hundred known venomous snakes belong to the following four families: atractaspidids, colubrids, elapids and viperids. The crotalids include pit vipers, moccasins and rattlesnakes while coral snakes belong to the elapids.

Snake venom contains biologically active ingredients with cytotoxic, neurotoxic and anticoagulant effects. From the cardiac system point of view, the phosphodiesterases that lower blood pressure and phospholipase A2 that causes hemolysis are most significant.

The subcutaneous LD<sub>50</sub> of cottonmouth (*Agkistrodon piscivorus*) venom is 25.8mg/kg body weight, for *Vipera berus* is 6.45mg/kg body weight and for *Vipera latifii* is 4.61mg/kg body weight. Disruption of the normal blood clotting pathways is significant. Some prevent clot formation, inducing hemorrhagic shock. Other toxins induce clotting, causing stroke and heart attacks by blocking blood flow.

Cardiotoxins bind to muscle cells causing depolarization and prevention of muscle contraction, thus interfering with the rhythm of the heart. Snake bites usually are noted in dogs bitten on the head or neck. In one particular study, cardiac variables were normal on auscultation at admittance, post-envenomation. Twenty-four hours later, 11% had cardiac abnormalities. Muscle damage as evidenced by elevated CK values in serum biochemistry was noted in 54% of sampled dogs (Segev *et al.*, 2008; Lervik *et al.*, 2010). The myocardial injury was verified by ECG performed post-envenomation. Myocardial damage post-envenomation was also shown by the increase of cardiac-specific troponin I.

Polyvalent antivenoms are available for the treatment of most pit viper bites. Coral snake bites require neurotoxic antivenom.

### Blister beetles

Blister beetles prefer alfalfa fields where large swarms tend to congregate. When these fields are harvested, the blister beetles are crushed and incorporated into the bales of hay. They contain a poisonous substance known as cantharidin. It is an inhibitor of the serine-threonine protein phosphatase. Cantharidin blocks the adenosine A<sub>1</sub> receptors, which regulate myocardial oxygen consumption, yielding anti-adrenergic effect in ventricular cardiac myocytes (Narayan *et al.*, 2000). Cantharidin can exert a positive inotropic effect in cardiac muscle by increasing calcium influx (Neumann *et al.*, 1995).

In equids, the LD<sub>50</sub> of cantharidin is reported to be 1 mg/kg body weight (Guglick *et al.*, 1996). A dose of 4 g of dried beetles is lethal to a horse and 1–1.5 mg/kg body weight is lethal to cats and dogs. The cardiac symptoms of cantharidin poisoning include increased heart rate and myocardial dysfunction.

There is no antidote for cantharidin but symptomatic treatment is recommended with administration of fluids and maintenance of serum electrolytes.

## CONCLUSIONS AND FUTURE DIRECTIONS

Cardiac problems in domestic animals are primarily due to toxic plants, chemicals and envenomation, most of which can be avoided. There is only a limited amount of published research on cases involving inadvertently affected animals. Animal cardiac responses have been extensively studied in the development of drugs. A considerable amount of work still needs to be done on cardiotoxicity in animals caused outside of drug development.

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## Liver toxicity

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### INTRODUCTION

The liver is a remarkable organ that usually protects the individual against injury from xenobiotic compounds. The liver is susceptible to injuries by chemical compounds because it is often the site of metabolism and it is where some chemicals concentrate and become bioactivated. Although its capacity for repair and regeneration (Ramaiah *et al.*, 2004; Mehendale, 2005) makes it a quite robust organ, if the ability to regenerate is not adequate, or if injury to the liver is very severe, liver damage may progress to liver failure and death.

There is continued interest concerning the higher incidence of liver damage caused by prescription/injectable drugs, over-the-counter medications or dietary supplements that are often combined with special diets and alcohol consumption, in addition to environmental chemicals/xenobiotics (Watkins, 1999). Though alcohol consumption is not a major risk factor in most veterinary patients, other factors such as toxic pasture or house plants, cyanobacterial toxins, pollutants, fungal toxins and toxicants in household waste are associated with hepatic injury. An incomplete list of hepatotoxins is found in Table 17.1. Complicating matters is the increasing population of geriatric veterinary patients. Preexisting liver disease must be considered in patients exposed to liver toxicants.

In humans, drug-induced liver injury has become the most frequent cause of acute liver failure in the United States and around the world, exceeding all other causes combined. Although the incidence of idiosyncratic drug-induced liver injury (DILI) with approved drugs at therapeutic doses is relatively low and estimated at 1 to 10

per 100,000 treated patients, the outcome is potentially a very grave one. Approximately 10% of those human patients will die or require liver transplantation, making idiosyncratic DILI the leading causes for urgent liver transplantation (Stirnemann *et al.*, 2010). Toxic liver disease also remains the single major cause for regulatory actions concerning drugs. Such actions may include failure of approval, withdrawal from the market, restrictions on use and warnings to physicians.

Liver injury by xenobiotics is also encountered in a variety of circumstances. Some natural toxins such as the cyclic peptides of *Amanita phalloides*, pyrrolizidine alkaloids, cycasin from cycad palms and other phytochemicals are examples of environmental hazards. They may be ingested by the curious or very hungry veterinary patient. Others, such as mycotoxins, are ingested unknowingly because of feed contamination due to climatic conditions favorable to fungal growth. A striking example of this phenomenon is the recent contamination of dog food with aflatoxin resulting in dozens of canine deaths in late 2005 and early 2006 (Stenske *et al.*, 2006; Newman *et al.*, 2007; Dereszyński *et al.*, 2008). Other circumstances of exposure to hepatotoxins in the home or farm include accidental food or feed contamination with industrial chemicals or pesticides. For example, in 1955, a human outbreak of hepatic porphyria in Turkey (Can and Nigogosyan, 1963) was caused by ingestion of wheat to which hexachlorobenzene, a fungistatic agent, had been added.

The goal of this chapter is to provide a basic understanding of the liver physiology and pathophysiology, and to expand on the common toxicosis inducing liver injury in veterinary medicine.

TABLE 17.1 Incomplete list of hepatotoxins of veterinary interest

Classification of mechanism	Toxicant	Source of toxicant	Source classification	Species affected	
Free radical	Carbon tetrachloride	Cleaning agents	Industrial	All	
	Yellow phosphorus	Fireworks	Industrial	All	
	Iron	Dietary supplement	Household	All	
	Copper	Dietary supplement	Feed, industrial	Sheep, some dog breeds	
Electrophile	Aflatoxins	<i>Aspergillus</i> spp.	Mycotoxin	All	
	Acetaminophen	NSAID	Household	All, cats	
	Sesquiterpene lactones	<i>Helenium</i> spp., <i>Hymenoxis</i> spp.	Plant	Herbivores	
	Pyrrolizidine alkaloids	<i>Amsinkia</i> spp.	Plant	Herbivores	
		<i>Crotolaria</i> spp.			
		<i>Cynoglossum</i> spp.			
		<i>Echium</i> spp.			
		<i>Erechtites glomerulata</i>			
		<i>Senecio</i> spp.			
	Biliary obstruction	Sporodesmin	<i>Pithomyces chartarum</i>	Mycotoxin	Herbivores, ruminants
Sapogenins		<i>Tribulus terrestris</i>	Plant	Herbivores, ruminants	
		<i>Panicum</i> spp.	Plant	Herbivores, ruminants	
		<i>Agave lecequilla</i>	Plant	Herbivores, ruminants	
		<i>Nolina texana</i>	Plant	Herbivores, ruminants	
		<i>Cycas</i> spp.	Plant	Dogs	
Other	Glycosides of methylazocymethanol				
	Carboxyatractyloside	<i>Xanthium</i> spp.	Plant	Swine, herbivores	
	Triterpenes	<i>Lantana</i> spp.	Plant	Herbivores	
	Amatoxins	<i>Amanita</i> spp.	Mushroom	All	
	Microcystins	<i>Microcystis</i> spp.	Cyanobacteria	All	
	Nodularins	<i>Nodularia</i> spp.	Cyanobacteria	All	
	Phenolics and coal tar derivatives	Disinfectants, clay pigeons	Household	All	
	Unknown	Joint supplements	Drug	Dogs	
	Idiosyncratic	Sulfonamides	Antimicrobial	Drug	Dogs
		Carprofen	Anti-inflammatory	Drug	Dogs
Diazepam		Sedative	Drug	Cats	

## STRUCTURAL AND FUNCTIONAL ORGANIZATION OF THE LIVER

### Lobule and acinus

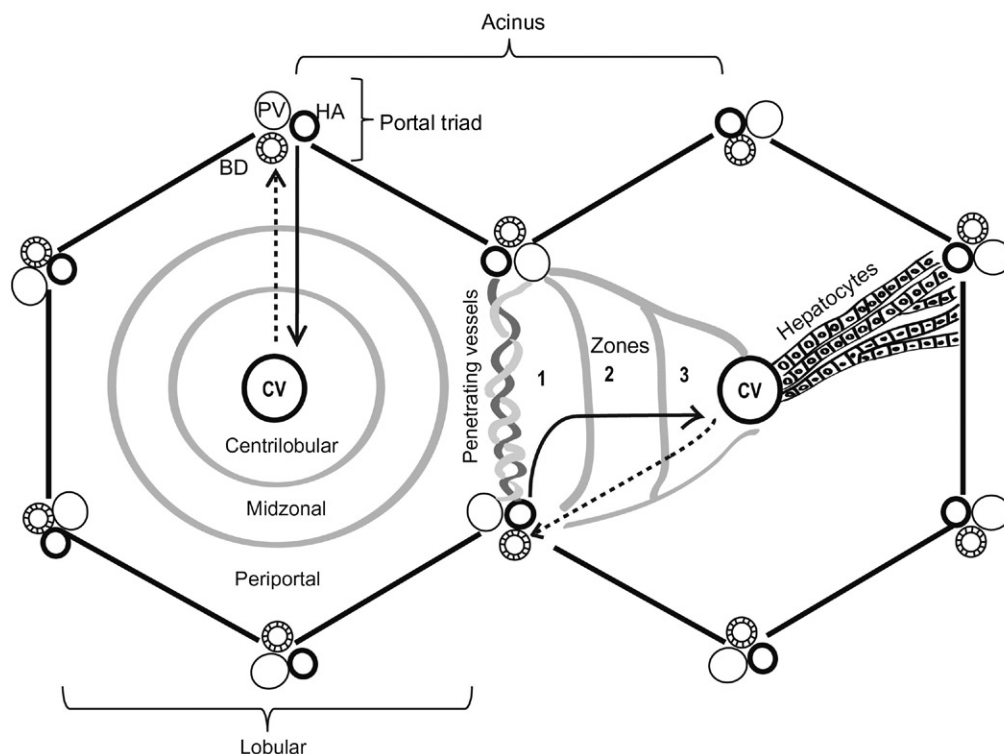
The structural and functional organization of the liver has been described by two concepts called the hepatic lobule and hepatic acinus models. The hepatic lobule, a more classical model, is defined histologically as a hexagonal region of parenchyma surrounding the central vein. Six portal triads, composed of branches from the portal vein, hepatic artery and bile ductules, border the edge of the lobule. One-cell thick plates or cords of hepatocytes are arranged radially around the central vein, forming the blood sinusoids between them. The hepatic parenchyma is divided into three zones based on proximity to the central vein (Figure 17.1, left). The area adjacent to the central vein is termed *centrilobular*, the area adjacent to the portal triad is *periportal*, and between the centrilobular and periportal parenchyma is the *midzonal* area.

The hepatic acinus, on the other hand, was defined by Rappaport as the smallest structural and functional unit in the liver based on the microcirculation in the liver, and is preferred as a concept of a functional hepatic unit (Figure 17.1, right). The blood in the liver flows from the portal vein and hepatic artery of the portal triad, is mixed in the penetrating vessels, enters the sinusoids, and eventually exits via central hepatic vein. The cells of the acinus are arbitrarily divided into three zones according to the sequence of the blood flow: *zone 1* corresponds to the periportal zone, *zone 2* corresponds to the midzonal area, and *zone 3* corresponds to the centrilobular zone.

### Diverse function of the liver

The liver is a functionally diverse organ. The liver is directly involved in (1) nutrient homeostasis, including glucose regulation (Treinen-Moslen, 2001; Pineiro-Carrero and Pineiro, 2004), cholesterol synthesis and uptake (Treinen-Moslen, 2001), storage of glycogen,





**FIGURE 17.1** Lobular versus acinar concept in liver architecture. The hepatic lobule is defined histologically as a hexagonal region of parenchyma surrounding the central hepatic vein. The hepatic acinus is a functional unit based on the microcirculation of the liver, and is preferred as a concept of a functional hepatic unit. The hepatic parenchyma is divided into three sections based on proximity to the central vein in the case of lobular model (centrilobular, midzonal, periportal), and according to the sequence of the blood flow in the case of acinar model (zones 1–3). Blood flows towards the central vein (solid line) and bile flow towards to bile duct (dotted line). CV: hepatic central vein, BD: bile duct, HA: hepatic artery, PV: portal vein.

lipids, minerals and vitamins (Plumlee, 2004), (2) synthesis of clotting factors, albumin, very low density lipoprotein and other proteins (Treinen-Moslen, 2001; Plumlee, 2004) and (3) metabolism and excretion of such things as hemoglobin breakdown products (Plumlee, 2004), steroid hormones (Brown, 2001) and xenobiotics.

Blood from the gastrointestinal tract, via the portal vein, is filtered for bacterial products, such as endotoxin and ammonia, and xenobiotics (Treinen-Moslen, 2001; Plumlee, 2004). Energy-dependent transport exists for certain xenobiotics and endogenous hormones. These include a group of multiple drug resistance p-glycoproteins that transport lipophilic cationic drugs, estrogens and phospholipids, as well as the canalicular multiple organic anion transporters involved in movement of molecules conjugated to glutathione, glucuronide and sulfate. Metal and mineral transport functions are important for mineral homeostasis and occur through facilitated diffusion and receptor mediated endocytosis across the sinusoidal membrane. Lysosomes within hepatocytes are involved in storage and export of metals and minerals into canaliculi.

Sinusoids are located between cords of hepatocytes, which are larger than capillaries and lined by

specialized endothelium. The endothelial lining is discontinuous, the sinusoidal endothelial cells having fenestrae, to allow movement of fluid and molecules less than 259 kDa into the space of Disse, located between the endothelium and the hepatocytes (Watkins, 1999; Treinen-Moslen, 2001; Plumlee, 2004). Within the space of Disse, hepatocytes contact free and protein bound molecules which may be absorbed by diffusion or aforementioned active transport (Watkins, 1999). Remarkable metabolic diversity of the hepatic zones is needed in order to accommodate the liver's numerous functions. Zone 3 hepatocytes are involved in glycolysis and lipogenesis, and zone 1 hepatocytes are involved in bile salt extraction, fatty acid oxidation, gluconeogenesis and protein synthesis and thus are rich in mitochondria for energy production (Treinen-Moslen, 2001; Pineiro-Carrero and Pineiro, 2004; Plumlee, 2004). Gradients of metabolic enzymes involved in bioactivation and detoxification have been shown along different zones of the acinus (reviewed by Jungermann and Katz, 1989). Zone 3 is particularly rich in phase I metabolic enzymes and zone 1 has highest glutathione levels involved in phase II metabolism.

The blood supply of the liver is mainly from the portal vasculature: 60 to 80% from branches of the portal vein, which supplies nutrients, drug substance and toxins from the gastrointestinal tract but is oxygen depleted, and 20 to 40% from the hepatic artery, which is oxygenated (Treinen-Moslen, 2001). Oxygen is quickly consumed to meet the high metabolic demands of the parenchymal cells as the blood flows towards the terminal hepatic venule, also known as central vein. As a consequent of this structure, hepatocytes in zone 1 receive blood that is 9 to 13% oxygenated whereas blood received by zone 3 is only 4 to 5% oxygenated and nutrient depleted (Sturgill and Lambert, 1997; Treinen-Moslen, 2001). Therefore zone 3 or the centrilobular zone has substantially lower oxygen concentrations compared to the rest of the tissue and is susceptible to hypoxia.

Another well-known gradient seen in acinar zonation is that of bile. Bile salts and bilirubin from blood enter hepatocytes via active transporters on the sinusoidal membrane, and are then secreted into canalicular lumen via canalicular membrane (Treinen-Moslen, 2001). Bile canaliculi are dynamic structures located between hepatocytes and formed by the hepatocyte membranes (Treinen-Moslen, 2001; Plumlee, 2004). Bile flows from hepatocytes, into canaliculi, and towards the direction of the bile duct of the portal triad in the liver acinus, from zone 3 to 1, in the opposite direction of the blood flow. Bile salts are efficiently extracted in zone 1 and there is little uptake by the time blood reaches zone 3.

Bile secretion is a major hepatic function essential for uptake of lipid nutrients, for protection against oxidative insults in the small intestine and for excretion of endogenous and xenobiotic compounds. Bile is composed of bile salts, bilirubin, glutathione, phospholipids, cholesterol, proteins, organic anions, metals and conjugated xenobiotics (Treinen-Moslen, 2001; Pineiro-Carrero and Pineiro, 2004).

Canaliculi enter canals of Hering in the portal triad that lead to interlobular and intrahepatic bile ducts, surrounded by bile duct epithelial cells, which coalesce to form the hepatic bile duct. Most animal species have a gall bladder for bile storage but others lack them, including horses and related species, rhinoceroses and tapirs, elephants, camelids, porcupines, pigeons, rats, deer, ratites and many parrots. Gall bladders may or may not be present in some species such as giraffes and hippopotamuses (Oldham-Ott and Gilloteaux, 1997). Most carnivores have a gall bladder, the exceptions being dolphins and other cetaceans.

The gall bladder stores and concentrates bile, then empties into the duodenum. When the gall bladder is absent, bile empties directly into the duodenum (Ramaiah, 2007). Bile enhances nutrient uptake in the small intestine, protects enterocytes from oxidation and facilitates excretion of xenobiotics and endogenous waste in the feces (Treinen-Moslen, 2001).

## Cell types of the liver

Seven intrinsic cell types have been identified in the liver: the parenchymal cells (or hepatocytes), the hepatocytic stem cells (oval cells) and five non-parenchymal cell types. Non-parenchymal cells include bile duct epithelial cells, sinusoidal endothelial cells, described above, as well as the resident macrophages (Kupffer cells), the large granular lymphocytes (pit cells) and the lipid storing hepatic stellate cells (HSC or Ito cells). Hepatocytes represent about 60% of the total cell number and 80% of hepatic tissue volume in the rat liver, and non-parenchymal cells are estimated to constitute about 30% of total cellular population, but only 6–7% of tissue volume due to their smaller size (Dahm and Jones, 1996).

Oval cells, the hepatocytic stem cells, are located in the canals of Hering, where bile canaliculi from the hepatic cords converge with bile ductules of the portal triad. If the hepatocyte replication is impaired, the oval cells proliferate and differentiate to eventually become both hepatocytes and biliary epithelial cells (Fausto and Campbell, 2003; Jaeschke, 2008). Experimental blockade of hepatocyte proliferation can potentially lead to continued oval cell proliferation leading to neoplastic transformation of hepatocytes resulting in hepatocellular tumors. New hepatocytes progress down hepatic cords to replace the aging zone 3 hepatocytes.

Kupffer cells represent 80% of the fixed macrophages in the body (Treinen-Moslen, 2001). These macrophages are usually located within sinusoids and are closely associated with endothelial cells. Kupffer cells are phagocytes, ingesting foreign material from the portal circulation (Treinen-Moslen, 2001; Plumlee, 2004) and debris from apoptotic or necrotic hepatocytes. Kupffer cells have other immune functions. They act as antigen presenting cells and secrete various cytokines (Treinen-Moslen, 2001; Plumlee, 2004), and are involved in destruction of metastatic neoplastic cells (Plumlee, 2004). Kupffer cells may store minerals and are involved in the pathogenesis of a variety of liver diseases induced by toxins such as ethanol (Laskin, 1990; Thurman, 1998).

Pit cells are natural killer cells with antineoplastic functions and are involved in granuloma formation and reside within sinusoids (Treinen-Moslen, 2001; Plumlee, 2004). Pit cells can proliferate locally when stimulated and attack tumor cells together with Kupffer cells (Wisse *et al.*, 1997).

Hepatic stellate cells (HSCs) or Ito cells are located in space of Disse and store fat and vitamin A (Treinen-Moslen, 2001; Pineiro-Carrero and Pineiro, 2004; Plumlee, 2004). When there is liver injury, HSCs are activated to myofibroblast-like cells (Plumlee, 2004; Maddrey, 2005). Activated stellate cells produce collagen and play a role in hepatic fibrosis (Treinen-Moslen, 2001; Plumlee, 2004).

## FACTORS INFLUENCING TOXIC LIVER INJURY

### Uptake and concentration

The liver has a dual blood supply as noted above. The hepatic artery brings blood from the systemic circulation and the portal system brings blood directly from the gastrointestinal system. The portal system is involved in the "first pass effect," meaning that nutrients and xenobiotics absorbed by the digestive system are filtered through the liver before entering the systemic circulation (Treinen-Moslen, 2001).

The space of Disse allows close contact between circulating plasma, plasma proteins and hepatocytes, allowing rapid diffusion of lipophilic compounds across the hepatocyte membrane (Treinen-Moslen, 2001). Some compounds are specifically taken up by sinusoidal transporters, including bile acids, phalloidin from several *Amanita* spp. of mushrooms, and microcystin produced by the cyanobacteria *Microcystis aeruginosa*.

Liver cells can accumulate high levels of metals and vitamins, leading to injury. For example, excessive storage of vitamin A in stellate cells produces engorgement, activation and proliferation of these cells (Treinen-Moslen, 2001). Chronic high vitamin E concentrations produce hepatic fibrosis and portal hypertension in humans, precipitating continued fibrosis (Zimmerman, 1999; Pineiro-Carrero and Pineiro, 2004; Maddrey, 2005). Cadmium is sequestered by hepatic metallothioneins but produces pathology when storage capacity is exceeded.

The liver is also responsible for iron homeostasis. Iron uptake from the sinusoids is receptor mediated and sequestration uses iron storage proteins such as ferritin. High hepatic iron concentrations produce lipid peroxidation affecting zone 1 hepatocytes (Treinen-Moslen, 2001). Certain bird species such as toucans, aracari, mynahs and birds of paradise often have hemosiderosis, accumulation of iron in the liver, and are prone to hemochromatosis, a disease state of iron build-up. Increased expression of iron transporters DMT1 and Ireg1 has been found in one of the susceptible species, mynahs, which are likely to be the cause of iron accumulation in this particular species (Mete *et al.*, 2005). However, the genetic cause(s) in mynahs or other susceptible avian species has not been completely determined yet.

The liver also plays an important role in copper homeostasis. Inability to export copper into bile is the cause of Wilson's disease in humans. This disease is caused by a defect or lack of an autosomal recessive gene called P-type ATPase (ATP7B), a copper transporter (Jaeschke, 2008). Similar copper storage disease of veterinary relevance, either mediating or as a result of chronic liver damage, is noted in certain breeds of dogs: Bedlington

terriers, West Highland white terriers, Skye terriers, Dalmatians, Doberman pinschers, Labrador retrievers, Anatolian shepherds, cocker spaniels, poodles and others (Rolfe and Twedt, 1995; Spee *et al.*, 2006). Recently, deletion of exon 2 of the MURR1 (COMMD1) gene, important in regulation of copper excretion into bile, was found to be the genetic defect found in Bedlington terriers (Klomp *et al.*, 2003), although molecular background of all other breeds are still unknown. Sheep are also susceptible to copper storage disease, which usually presents acutely.

Ability to excrete a toxicant into the biliary duct will usually result in clearance by excretion in feces or urine. However, certain lipophilic compounds such as methylmercury and diclofenac are commonly reabsorbed from the intestinal lumen back into the system, a process known as *enterohepatic cycling*. This will lead to long biological half-life and increased toxicity of the compound (Jaeschke, 2008).

### Bioactivation and metabolism

One of the major functions of the liver is elimination of both endogenous and exogenous compounds (xenobiotics). Most xenobiotic agents absorbed by the small intestine are highly lipophilic. Renal excretion is the primary mechanism of removal for many xenobiotics, but renal excretion of lipophilic compounds, which are frequently protein bound in the circulation, is poor (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins, 1999). In order for these xenobiotics to be eliminated, biotransformation needs to occur in the liver first to increase their water solubility for excretion.

Hepatocytes contain high levels of phase I enzyme, which exposes or introduces functional groups ( $-OH$ ,  $-NH_2$ ,  $-SH$  or  $-COOH$ ) and generates reactive electrophilic metabolites. They also have a wide variety of phase II enzymes which enhance the hydrophilicity by adding a polar group by conjugation reaction to allow excretion. The functional groups exposed or introduced by the phase I reaction often become the site of action for the phase II reaction. These reactions often function in the detoxification of compounds, but there is significant potential for accumulation of toxic metabolites if the phase I and II reactions are not appropriately balanced (Zimmerman, 1999; Jaeschke, 2008).

Phase I reactions, ubiquitous in mammals, involve oxidation, reduction and hydrolysis, and sometimes produce biologically active metabolites (Brown, 2001). Phase I enzymes are predominantly located in zone 3, as mentioned previously. Many of these phase I enzymes are present in the smooth endoplasmic reticulum of the hepatocyte. When liver tissue is homogenized mechanically in a laboratory, the endoplasmic reticulum breaks

down into small vesicles known as microsomes and can be retrieved by differential centrifugation. Thus these enzymes are often referred to as microsomal enzymes. As a rule, microsomal enzymes require oxygen and NADPH to function (Dahm and Jones, 1996; Brown, 2001).

Oxidation is the major phase I reaction. Important substrates include steroid hormones and lipid soluble drugs (Brown, 2001). Oxidative reactions can produce highly reactive epoxides, which are usually detoxified rapidly by phase II conjugation or hydrolysis by microsomal epoxide hydrolase enzymes (Watkins, 1999; Pineiro-Carrero and Pineiro, 2004). If an individual is exposed to the xenobiotic in high enough doses that these mechanisms are overwhelmed, reactive phase I metabolites produce injury.

The cytochrome P450 enzymes, a major group of the microsomal enzymes, are the most common players of phase I oxidation. All P450 enzymes contain a heme iron center and when complexed within carbon monoxide, absorb light at a wavelength of 450nm. These enzymes were first discovered in an unknown pigment thus named cytochrome P450: P for pigment and 450 for the wavelength of absorbed light. Liver P450 enzymes involved in xenobiotic biotransformation belong to three main P450 gene families, CYP1, CYP2 and CYP3. More than 36 cytochrome P450 isoenzymes have been identified in animals (Dahm and Jones, 1996; Watkins, 1999; Jaeschke, 2008). P450s can be induced or inhibited by drugs such as phenobarbital and cimetidine, respectively.

Non-cytochrome P450 enzymes are also involved in oxidative reactions. One such enzyme is alcohol dehydrogenase, a cytosolic enzyme, whose substrates include vitamin A, ethanol and ethylene glycol. Others include aldehyde dehydrogenase, aldehyde oxidase, xanthine oxidase, monoamine oxidase, diamine oxidase, peroxidase and flavin-monooxygenases (Jaeschke, 2008).

Examples of phase I reduction include nitro compounds, which are reduced to amines, and volatile anesthetics, which undergo dehalogenation. Phase I hydrolysis reactions are required for metabolism of compounds with amide bonds or ester linkages, as in the conversion of aspirin to salicylate (Brown, 2001). Carboxylesterase, alkaline phosphatase, peptidase and epoxide hydrolase are involved in phase I hydrolysis reactions (Jaeschke, 2008).

Most phase II enzymes are cytosolic but others are mitochondrial or microsomal. Phase II enzymes are predominantly involved in conjugating xenobiotics at the functional groups of the parent compound or the phase I metabolites. Unlike the phase I metabolites, phase II metabolites are rarely reactive, with some exceptions such as the glucuronide of some nonsteroidal anti-inflammatory drugs and the aromatic amines (Jaeschke, 2008), which will be discussed later.

Phase II enzymes conjugate a polar group to a hydroxyl-, carboxyl-, amino- or sulfhydryl-group on the substrate. Polar molecules added to substrates include glucuronic acid, sulfate derived from sulfuric acid ester, acetate, glutathione, methyl groups derived from methionine and amino acids such as glycine and cysteine. These groups enhance water solubility to allow for renal or biliary excretion of the metabolite. Disruption of phase II reactions allows accumulation of reactive phase I metabolites (Sturgill and Lambert, 1997).

Glucuronidation is the most common phase II reaction in humans, though it is deficient in the neonate and, of significant veterinary importance, in cats (Sturgill and Lambert, 1997; Brown, 2001; Pineiro-Carrero and Pineiro, 2004). Substrates with a molecular weight greater than 500, such as steroid hormones, thyroxine, bilirubin, salicylates, acetaminophen and many other drugs, are likely to undergo glucuronide conjugation. Glucuronyltransferases are microsomal enzymes that catalyze the transfer of glucuronide from uridine 5'-diphosphate (UDP). UDP becomes depleted in overdoses of acetaminophen and other drugs that undergo this detoxification pathway. Products of glucuronide conjugation are excreted in the bile or urine. Those excreted in the bile sometimes undergo hydrolysis in the intestine, leading to *enterohepatic cycling*. Phenobarbital is one agent that induces glucuronyltransferases.

Sulfation is the primary conjugation reaction for aliphatic alcohols and substrates with phenol groups (Sturgill and Lambert, 1997; Brown, 2001). These reactions are catalyzed by cytoplasmic sulfotransferases. Substrates for sulfate conjugation include acetaminophen, morphine, ascorbic acid and endogenous compounds like chondroitin, heparin and some steroid hormones. The pool of available sulfates may become saturated in drug overdoses.

Drugs with amine and hydrazine groups, such as sulfonamides, are often conjugated to acetate (Sturgill and Lambert, 1997; Brown, 2001). N-acetyltransferase is a cytoplasmic enzyme involved in acetylation reactions.

Glutathione (GSH) and cysteine have sulfhydryl groups which readily bind many phase I metabolites (Brown, 2001). GSH, a free radical scavenger, prevents membrane damage from reactive metabolites in spontaneous reactions or with the selenium-dependent GSH peroxidases as a catalyst. Because these enzymes are cytosolic, damaged membrane phospholipids must be released by phospholipase A2 for detoxification. GSH is also involved in reduction and recycling of other antioxidants such as vitamins E and C (Dahm and Jones, 1996). When oxidized, GSH forms a dimer which must be reduced by NADPH-dependent GSH reductases. GSH becomes depleted in the fasting or overdosed patient (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Pineiro-Carrero and Pineiro, 2004). N-acetylcysteine is used to replenish GSH.



Significant species differences exist in phase II enzymes. UDP-glucuronosyltransferase is deficient in cats, affecting their ability to detoxify numerous compounds including acetaminophen (Court and Greenblatt, 1997; Brown, 2001). The Gunn rat, a mutant strain of Wistar rat, is also deficient in UDP-glucuronosyltransferase enzyme. Ferrets also have less capability to glucuronidate (Court, 2001). Swine have reduced sulfate conjugation abilities (Brown, 2001). Dogs do not have the ability to acetylate aromatic groups, which may interfere with their ability to metabolize sulfonamide antimicrobials (Brown, 2001; Trepanier, 2004).

Reactive metabolites of phase I act as free radicals, substances with unpaired electrons. Free radicals generate reactive oxygen species such as superoxide ( $O_2^{\bullet-}$ ), hydroxyl radicals ( $\bullet OH$ ) and hydrogen peroxide ( $H_2O_2$ ) through interactions with other molecules. Free radicals reacting with phospholipids of the plasma membrane, endoplasmic reticulum membrane or mitochondrial membranes cause a self-propagating chain reaction leading, eventually, to cell death (Dahm and Jones, 1996; Watkins, 1999).

Epoxides and other electrophiles are molecules with electron seeking properties. These form covalent bonds with nucleophilic molecules, for example by binding thiol groups on proteins (Dahm and Jones, 1996; Watkins, 1999; Zimmerman, 1999). Electrophiles produced by phase I reactions cause cell death by damage to critical proteins such as calcium transport proteins in membranes. Aflatoxin  $B_1$  binds to guanine residues in the DNA, leading to defects in protein transcription, resulting in carcinogenesis or cell death.

Aromatic amines and NSAIDs are examples where phase II reaction can form reactive metabolites, as mentioned previously. Glucuronidation of N-hydroxyaromatic amines, metabolites of aromatic amines, lead to formation of highly electrophilic aromatic nitrenium ions that can bind to DNA and other macromolecules, leading to bladder cancer. Several drugs including NSAIDs can form acyl-glucuronides, reactive intermediates, which bind to proteins to form adducts.

In addition to phase I and II biotransformation enzymes, studies suggest the involvement of hepatic transporter systems in drug efflux from hepatocytes as a means for the liver to rid itself of foreign chemicals. These are called the phase III transporter systems. Several transporter families mediating uptake of chemicals into liver and excretion of chemicals from liver into blood and/or bile have been identified. Generally, the organic anion transporting polypeptide family (OATPS), organic cation transporter 1 (OCT1) and organic anion transporter 2, mediate uptake of a large number of xenobiotics from the blood to the liver. Conversely, multidrug resistance proteins (MDRS), multidrug resistance associated proteins (MRPS) and breast cancer resistance

protein (BCRP) mediate efflux of xenobiotics from liver into bile or blood (Klaassen and Slitt, 2005). Similarly to phase I and II enzymes, these transporters can be induced or inhibited by drugs that are ligands of various nuclear receptors, such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane x receptor (PXR).

## Inflammation

Inflammatory reaction represented by activation of resident Kupffer cells, natural killer and natural killer T cells and migration of activated neutrophils, lymphocytes and monocytes in the damaged liver region is a very common characteristic of toxin-induced hepatotoxicity. Although the main role of this inflammatory response is to remove dead and damaged cells, it can also aggravate the injury by releasing or forming cytotoxic, pro- and anti-inflammatory mediators (Jaeschke, 2008).

Upon activation, Kupffer cells generate reactive oxygen species (ROS), such as hydrogen peroxide, by the action of NADPH oxidase. These ROS will diffuse into neighboring hepatocytes, pose oxidative stress and lead to cell injury. It has also recently been found that intracellular proteins such as high-mobility group protein 1 (HMGB-1) are released by cells during necrosis, bind to toll-like receptors on Kupffer cells, which induces the synthesis and release of cytokines and chemokines (such as  $TNF-\alpha$ , IL-1). This leads to recruitment of cytotoxic neutrophil which can directly cause apoptotic cell death or generate ROS, such as hypochlorous acid, by the actions of NADPH oxidase and myeloperoxidase, and lead to cell injury (Jaeschke, 2008).

The role of Kupffer cells in toxicant-induced liver injury has been suggested by a variety of chemicals such as ethanol, acetaminophen,  $CCl_4$  and 1,2-dichlorobenzene. Involvements of neutrophils have been shown with hepatotoxicity associated with alpha-naphthylisothiocyanate and halothane. Although many compounds such as ethanol, allyl alcohol, aflatoxin  $B_1$ , monocrotaline, ranitidine and diclofenac are capable of causing liver injury without the involvement of neutrophils, inflammatory response initiated by endotoxin triggers a neutrophil-induced injury or aggravates the existing injury.

## TYPES OF TOXICANT-INDUCED LIVER INJURY

### Cell death

Necrosis is the predominant form of cell death in most toxic insults. Microscopically visible degenerative

changes to the hepatocyte may precede necrosis, including ballooning degeneration, hyaline degeneration and the presence of Mallory bodies (Zimmerman, 1999). Cells lose osmotic homeostasis and swell, which is visible on light microscopy. Swelling of organelles is seen on an ultrastructural basis (Dahm and Jones, 1996; Treinen-Moslen, 2001). Energy production fails due to loss of calcium homeostasis (Dahm and Jones, 1996; Zimmerman, 1999). Eventually there is rupture of the cell membrane and leakage of cell contents. Typically, necrosis results in elevation of serum transaminases such as alanine transaminase, aspartate transaminase and sorbitol dehydrogenase. Depending on the extent of liver necrosis, liver function may or may not be affected.

Necrosis is often initiated by damage to membranes, either the plasma membrane of the cell or the membranes of organelles, particularly the mitochondria, such as with acetaminophen toxicosis. Cell membrane damage can be caused by peroxidation of membrane phospholipids, such as with carbon tetrachloride. Damage to the plasma membrane interferes with ion regulation, damage to the membranes of the mitochondria interferes with calcium homeostasis and energy production, and damage to the smooth endoplasmic reticulum membrane diminishes the ability of that organelle to sequester calcium (Zimmerman, 1999). Inhibition of protein synthesis is an alternate mechanism of cell necrosis. Toxins that act in this way include phalloidin and related mushroom toxins, which inhibit the action of RNA polymerase and therefore mRNA synthesis (Pineiro-Carrero and Pineiro, 2004).

Necrotic liver injury can be focal, zonal, bridging or massive and panlobular. Focal necrosis is randomly distributed and involves hepatocytes individually or in small clusters (Treinen-Moslen, 2001). Zonal necrosis is common and usually occurs in zone 3, the centrilobular area (Zimmerman, 1999; Treinen-Moslen, 2001; Plumlee, 2004) due to higher levels of phase I enzymes in this region. Grossly, the liver will have a reticulated pattern with dark red central areas separated by brown to yellow areas. Bridging necrosis describes confluent zones of necrosis that extend between zones of the lobule and between lobules (Treinen-Moslen, 2001). Panlobular or massive necrosis denotes hepatocyte loss throughout the lobule with loss of lobular architecture. The liver is grossly swollen and friable with panlobular injury.

Apoptotic cells undergo cell shrinkage, nuclear condensation and pyknosis (Dahm and Jones, 1996; Treinen-Moslen, 2001), but mitochondrial function (Pineiro-Carrero and Pineiro, 2004) is maintained and the cell membrane remains intact (Zimmerman, 1999). This type of cell death is not associated with inflammation. Although apoptosis, sometimes termed "programmed cell death," is a normal physiological process, it may be induced by xenobiotics due to oxidative stress, decrease

in apoptotic suppressors or enhanced expression of apoptosis genes. Apoptotic cells are occasionally seen in the centrilobular area but are rapidly phagocytized by macrophages and other hepatocytes.

Although necrosis and apoptosis are considered separate entities, an alternate view is emerging that apoptosis and necrosis are frequently the consequence of the same initiating factors and signaling pathways. Rather than being separate entities, apoptosis and necrosis in their pure form may represent extremes on a continuum of cell death. Thus necrosis resulting from oncosis is termed "oncotic necrosis" and that originating from apoptosis is termed "apoptotic necrosis" (Levin *et al.*, 1999; Jaeschke *et al.*, 2004; Malhi *et al.*, 2006).

The nature of the lesions can tell us something about the mechanism of injury. Several drugs/chemicals such as carbon tetrachloride (CCl<sub>4</sub>), acetaminophen, thioacetamide, allyl alcohol and ethanol result in hepatocyte oncotic necrosis. Centrilobular necrosis is a common pattern and is seen with carbon tetrachloride, acetaminophen and thioacetamide. Hypoxia also causes centrilobular necrosis since this region, or zone 3, has lowest levels of oxygen, as mentioned previously.

Less commonly, a midzonal pattern is seen in endotoxin-mediated hepatitis in a rat model (Ramaiah *et al.*, 2004). Necrosis of periportal hepatocytes can be observed, although rare, with compounds such as allyl alcohol. Hepatocytes in this region are usually young cells and necrosis often leads to portal fibrosis. Bile duct proliferation may also follow. Ethanol ingestion produces multifocal, random hepatic necrosis.

Necrosis may be a primary event caused by reactive intermediates (e.g., acetaminophen, thioacetamide and carbon tetrachloride) or may be a secondary event following infiltration of inflammatory cells as seen with ethanol.

## Hepatic steatosis

Hepatic steatosis/lipidosis or fatty liver is simply the accumulation of fat vacuoles within hepatocytes. Steatosis is a common response to a variety of hepatotoxins and represents a potentially reversible hepatocyte injury (Treinen-Moslen, 2001). Grossly, the affected liver will be swollen with rounded edges, friable and light brown to yellow in color. Due to the fat accumulation, sections of the affected liver will float in formalin (Plumlee, 2004). Compounds that produce prominent steatosis associated with mortality include the antiepileptic drug valproic acid and the antiviral agent fialuridine. Other toxins that may cause hepatic steatosis include aflatoxin, white or yellow phosphorus, ethanol and carbon tetrachloride.

Although steatosis has been considered a benign and reversible change, studies have determined association

to biochemical alterations that produce steatohepatitis, fibrosis and cirrhosis (Ramaiah *et al.*, 2004). Non-alcoholic fatty liver disease (NAFLD), a syndrome associated with obesity in humans, who are often type II diabetics, has a marked inflammatory component (Diehl, 2002). Several endocrine abnormalities also result in steatosis, making determination of a cause challenging. Serum transaminase concentrations in patients with steatosis are variable and often include a mild to moderate increase in serum transaminase. Cholestasis occurs with marked lipid accumulation.

Steatosis is termed microvesicular if fat vacuoles are small and do not displace the nucleus. Microvesicular steatosis is often associated with slow lipid accumulation (Bastianello *et al.*, 1987; Plumlee, 2004). This form of steatosis may indicate deficiency in mitochondrial  $\beta$ -oxidation of fatty acids. It is a relatively severe form of steatosis and has been seen in primates and dogs with aflatoxicosis (Bastianello *et al.*, 1987; Zimmerman, 1999) and people with valproic acid toxicosis (Sturgill and Lambert, 1997; Zimmerman, 1999).

Macrovesicular steatosis, more common in domestic animals, describes hepatocytes containing one, or occasionally a few, large fat vacuoles that displace the nucleus to the periphery of the cytoplasm (Plumlee, 2004). Macrovesicular steatosis indicates an imbalance between fatty acid uptake and secretion of very low density lipoproteins due to increased triglyceride mobilization, decreased fatty acid oxidation, decreased synthesis of very low density lipoproteins or other metabolic anomalies (Sturgill and Lambert, 1997; Zimmerman, 1999; Treinen-Moslen, 2001; Plumlee, 2004). Macrovesicular steatosis has been reported in dogs with subacute aflatoxicosis (Bastianello *et al.*, 1987). Zone 1 macrovesicular steatosis is reported with yellow phosphorus toxicosis (Zimmerman, 1999). Macrovesicular steatosis occurs with a variety of metabolic disease in domestic animals, particularly in obese cats with anorexia and preparturient cattle due to mobilization of lipids.

## Steatohepatitis

Steatohepatitis is usually the next stage of steatosis if untreated (Lieber, 1994; Bautista, 2002; French, 2003). Steatohepatitis is the accumulation of lipid and the presence of inflammatory cells within hepatic parenchyma. The inflammatory cells involved are usually neutrophils and mononuclear leukocytes. There is no specificity noted with this pathologic stage and the distribution of lipidosis and inflammation is random and multifocal. Conditions usually associated with steatohepatitis are alcoholic liver disease, NAFLD and endotoxemia secondary to intestinal disease. Any toxic compounds that cause steatosis can also result in steatohepatitis if the

condition is left untreated. It should also be noted that steatohepatitis in humans may progress to fibrosis/cirrhosis and hepatocellular carcinoma if the inciting cause is not removed or treated (Diehl, 2002). The clinical biochemistry alterations of the liver usually include elevated serum transaminases.

## Hepatic fibrosis

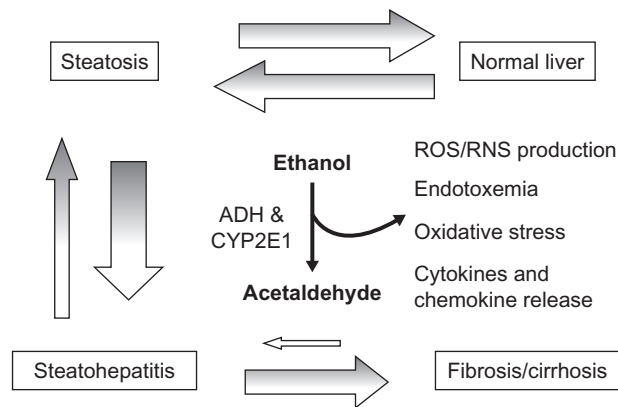
Fibrosis is a nonspecific lesion which usually results from chronic inflammation. Chronic inflammation can be the result of continuous exposure to a variety of hepatotoxic chemicals such as organic arsenicals, vinyl chloride or high doses of vitamin A (Zimmerman, 1999), chronic ethanol ingestion and non-alcoholic fatty liver disease. Fibrosis usually occurs around the portal area, in the space of Disse, and around the central veins. The hepatocytes are replaced with fibrous material and thus there is hepatocyte loss. Periportal fibrosis may lead to portal hypertension.

## Cirrhosis

Hepatic cirrhosis is end stage liver disease. Cirrhosis describes irreversible changes characterized by accumulation of excessive collagen deposition in the form of bridging fibrosis, disrupting hepatic architecture. Entrapped hepatocytes undergo random mitosis and growth, termed nodular regeneration. Cirrhosis may be micronodular or macronodular, depending on the amount of fibrosis and tissue regeneration. A liver with micronodular fibrosis has a grossly cobblestoned surface and is firm and difficult to cut with a knife.

The serum concentration of transaminase enzymes is low at this stage due to the lack of functional hepatocytes. Liver function parameters such as bile acids and ammonia are markedly elevated. Prognosis for recovery is poor. Physicians resort to liver transplantation as the only solution to restore adequate liver function in human patients.

Investigations of animals dosed with ethanol for one to several months have shown that the mechanisms of liver injury are numerous, indicating a complex, multifactorial pathogenesis for alcoholic liver disease, as seen in Figure 17.2 (Lieber, 1994). The roles of CYP2E1, fatty acid metabolism, oxidative damage, endotoxin, Kupffer cell and neutrophil infiltration have been extensively investigated (Di Luzio, 1966; Bardag-Gorce *et al.*, 2000; Kono *et al.*, 2000; Hoek and Pastorino, 2002). Progression of liver disease correlates well with the dose of ethanol consumed daily and the duration of alcohol consumption. Alcoholic hepatic steatosis, steatohepatitis, fibrosis and cirrhosis represent a sequential progression in



**FIGURE 17.2** Progression of alcoholic liver disease. Consumption of ethanol produces hepatic pathology in a sequence ranging from steatosis (fatty liver) on one extreme to fibrosis/cirrhosis on the opposite end of the spectrum. Steatosis and steatohepatitis represent acute stages of alcoholic liver disease. ROS = reactive oxygen species; RNS = reactive nitrogen species.

alcoholic liver disease. Females are more sensitive than males and experience a higher incidence of liver injury (Apte *et al.*, 2005). Inflammation is predominantly neutrophilic within regions of necrosis and Mallory body formation. Although many of these changes have been observed in experimental models of alcoholic hepatitis, progression to cirrhosis has rarely been experimentally reproduced.

### Pigment accumulation

Various substances can accumulate within hepatocytes or Kupffer cells and are often microscopically visible as pigment. Occasionally, these pigments lend a grossly visible tint to the liver. If there is damage to the biliary tract, by cholestasis or biliary obstruction, bile pigment can accumulate in canaliculi and bile ducts, producing a grossly yellow to green color (Zimmerman, 1999; Plumlee, 2004). If there is a bile canaliculus rupture, yellow pigment can be seen in hepatocytes and Kupffer cells.

Iron in the form of hemosiderin is stored in the liver as a yellow-brown pigment, which is better visualized using Pearl's Prussian blue (Plumlee, 2004). Copper is yellow-brown and is visualized using rhodanese. These accumulations of copper and iron can be released by the hepatocytes through necrosis. The newly regenerated hepatocytes may not have excessive levels and may show little pigment accumulation, thus copper toxicosis should be confirmed with kidney copper levels in such cases.

Lipofuscin can be present as a yellow-brown pigment within hepatocytic lysosomes as a senile change and as a result of cells being unable to break down old and damaged organelles in the cytosols (Plumlee, 2004). Melanin pigments can also be present in the liver hepatocytes,

Kupffer cells and the portal connective tissue without any disease or toxic insults. Differentiation of some of these pigments may be difficult histologically but can be easily done by use of special stains as mentioned above.

### Hepatic neoplasia

Chemically induced tumors can originate from hepatocytes, biliary epithelium and very rarely from sinusoidal endothelium. Neoplasms occur months or years after toxin exposure (Plumlee, 2004). If a toxin produces direct damage to DNA, a single exposure may lead eventually to neoplastic changes (Zimmerman, 1999; Pineiro-Carrero and Pineiro, 2004). Aflatoxin B<sub>1</sub> is an agent that acts by alkylating DNA and is associated with hepatocellular carcinoma in humans infected with hepatitis B virus. Aflatoxin B<sub>1</sub> has also been implicated in liver cancers in trout and laboratory animals (Newberne and Butler, 1969). Nongenotoxic agents must be given at high doses for long periods of time to induce cancer. Examples of nongenotoxic agents include phthalate esters in plasticizers, phenoxy acid herbicides and hypolipidemic drugs.

Hepatic adenomata are benign hepatocytic neoplasms associated with contraceptive steroid use in humans (Zimmerman, 1999). Hepatocellular carcinomata are malignant neoplasms induced by various chemicals and botanical toxins and are of more concern. They have been found in humans affected with hepatitis B virus and exposed to aflatoxin B<sub>1</sub>, as noted above, and associated with anabolic steroid abuse. Other risk factors include viral hepatitis C and D, ethanol abuse and exposure to microcystin.

Biliary carcinomata or cholangiocarcinomata are uncommonly associated with exposure to drugs/chemicals. Biochemical indicators of note include normal, elevated or low concentrations of serum transaminases, loss of liver function and the resulting increase in bile acid and ammonia concentrations.

Angiosarcomata or hemangiosarcomata derive from sinusoidal epithelium (Zimmerman, 1999; Treinen-Moslen, 2001; Plumlee, 2004). These neoplasms are rare but rapidly lethal and have been associated with exposure to vinyl chloride, inorganic arsenics and Thorotrast, a form of radioactive thorium dioxide once used in radiographic contrast studies.

### Megalocytosis

Hepatic megalocytosis can be observed in some aged animals but is also characteristic lesions observed by toxic insults, namely pyrrolizidine alkaloids and aflatoxins. It is caused by impaired cell division, and results



in enlarged hepatocytes with markedly enlarged nuclei (Plumlee, 2004). Pyrrolizidine alkaloids have an anti-mitotic effect on the hepatocytes without the inhibition of DNA synthesis. Since it is a natural urge for hepatocytes to proliferate to replace the damaged liver, hepatocytes become active in DNA and protein synthesis; however, they cannot divide and result in megalocytosis. Very often, chronic pyrrolizidine intoxication is accompanied by hepatic fibrosis as well as biliary hyperplasia and sometimes nodular regeneration of parenchyma.

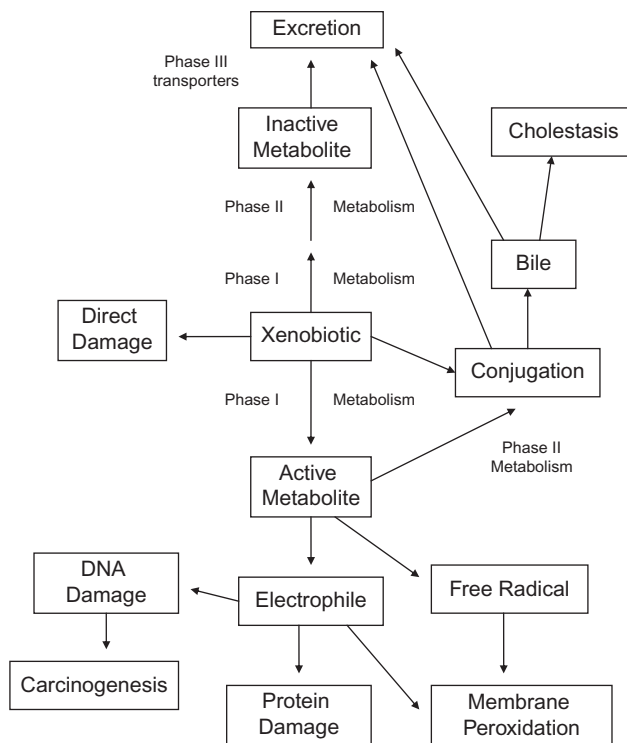
## MECHANISMS OF LIVER DAMAGE

Mechanisms of liver injury have been classified as either intrinsic or idiosyncratic. Intrinsic injury can produce steatosis, necrosis, cholestasis or a multiple of these lesions, often with minimal inflammation (Sturgill and Lambert, 1997). Intrinsic liver injury is a predictable, reproducible, dose-dependent response to a xenobiotic (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman, 1999; Pineiro-Carrero and Pineiro, 2004). A threshold dose exists for xenobiotics causing intrinsic liver injury and there is often a predictable latent period between the time of exposure and clinical evidence of liver injury. Intrinsic liver injury accounts for the vast majority of toxic liver injury cases and is often produced by reactive products of xenobiotic metabolism such as electrophiles or free radicals, though a few drugs cause intrinsic liver injury without bioactivation. An abbreviated summary of mechanisms of intrinsic liver injury is illustrated in Figure 17.3.

Idiosyncratic responses are, by contrast, unpredictable responses to a drug or other xenobiotic. They are rare, non-dose dependent and often associated with extrahepatic lesions (Sturgill and Lambert, 1997; Zimmerman, 1999; Pineiro-Carrero and Pineiro, 2004; Shenton *et al.*, 2004). Extrahepatic clinical signs may include pyrexia, rash and peripheral eosinophilia. Some idiosyncratic drug reactions resemble serum sickness. Hepatic changes associated with idiosyncratic drug reactions include necrosis, cholestasis or both, and there is often an inflammatory response involving macrophages and eosinophils.

### Oxidative stress mediated by free radicals

Free radicals are generated from within hepatocytes through a variety of mechanisms: ionizing radiation, oxidative metabolism by cytochrome P450s, reduction and oxidation (redox) reactions that occur during normal metabolism, transition metals such as iron and copper, and nitric oxide generated by a variety of inflammatory

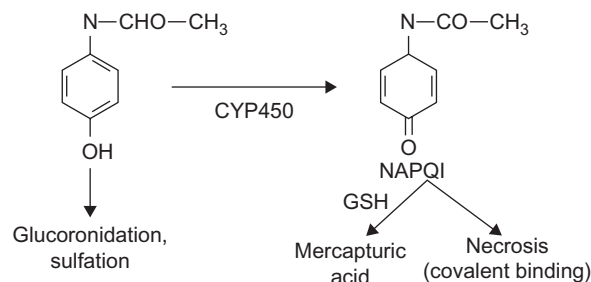


**FIGURE 17.3** Basic mechanisms of hepatic injury showing the relationship between multiple pathways for metabolism and toxicity for any compound. The liver metabolizes xenobiotics (and some endogenous compounds) to form water soluble products appropriate for urinary or biliary excretion. Some compounds are activated through these metabolic processes to free radicals, electrophiles or other toxic products that may induce hepatic injury.

cells. The reactive species generated result in lipid peroxidation of membranes, oxidative modification of proteins and DNA disruption (Crawford, 1999).

Free radicals have unpaired electrons, making them highly reactive. They may be formed by one electron oxidation and reduction reactions, producing cationic and anionic radicals, respectively (Dahm and Jones, 1996). Alternately, homolytic bond scission produces neutral radical formation. The free radical nitric oxide ( $\bullet\text{NO}$ ), an important cell signaling agent released by leukocytes, reacts with superoxide to form peroxynitrite. Hydroxyl radicals, superoxide radicals and hydrogen peroxide are major reactive oxygen species (ROS).

Free radicals produce peroxidation of phospholipids within the cell plasma membrane and in membranes of mitochondria and endoplasmic reticulum. The radicals remove a proton ( $\text{H}^+$ ) from a methylene carbon within a polyunsaturated fatty acid, forming a lipid-free radical. This step is called *initiation*. This lipid-free radical then abstracts a proton from a neighboring polyunsaturated fatty acid, generating more lipid-free radicals. *Propagation* by this mechanism is estimated to occur 4 to 10 times per initiation. The effects of lipid peroxy



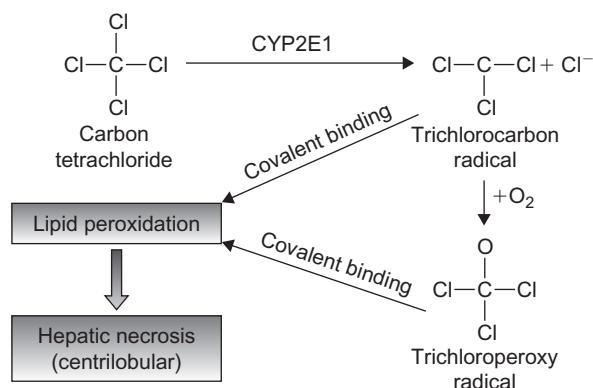
**FIGURE 17.4** Metabolism and mechanism of acetaminophen toxicity. Bioactivation of acetaminophen by P450 enzymes results in the formation of the reactive intermediate (NAPQI) which forms covalent adducts with glutathione which is then converted to mercapturic acid. When the amount of the reactive metabolite formed exceeds the glutathione available for binding, the excess metabolite binds to tissue molecules, forming covalent adducts, resulting in centrilobular hepatic necrosis.

radicals on the cell membrane include: increased permeability, decreased fluidity, inactivation of membrane proteins (Dahm and Jones, 1996) and, in the case of mitochondrial membranes, loss of polarity (Watkins, 1999). Lipid peroxy radicals react with metal ions stored within the hepatocyte to generate more lipid radicals.

The most frequent mechanism of free radical production associated with hepatocellular injury is bioactivation of xenobiotics by the cytochrome P450 system. Phase I metabolism activates substrates to reactive intermediate molecules in preparation for phase II conjugation reactions. However, in circumstances of high exposure, phase I products accumulate. Lesions produced by these compounds tend to be centrilobular due to the centrilobular location of the cytochrome P450s responsible for metabolism.

A classical example of phase I bioactivation is cell death resulting from acetaminophen (Figure 17.4). Acetaminophen is a widely used analgesic that rarely induces clinical signs at therapeutic doses, except in particularly susceptible feline species, where metabolites of acetaminophen produce acute liver failure and oxidation of hemoglobin to methemoglobinemia. The details of acetaminophen toxicity are described in another chapter in this book on over-the-counter drugs, but the unstable intermediate metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), is known to be the cause of hepatocyte injury.

The reaction that produces NAPQI generates superoxide anions as a by-product. NAPQI itself also acts as an electrophile, targeting mitochondria in particular, forming covalent adducts with protein thiol groups and other cellular macromolecules. Interaction of NAPQI with other cellular molecules also generates reactive oxygen species, leading to oxidative stress on the hepatocyte (Dahm and Jones, 1996; Zimmerman, 1999). Plasma membrane proteins involved in calcium homeostasis and



**FIGURE 17.5** Metabolism and mechanism of carbon tetrachloride toxicity. Carbon tetrachloride metabolism by CYP450 leads to free radicals such as trichlorocarbon and trichloroperoxy radical that initiate lipid peroxidation. The centrilobular location of CYP2E1 enzyme is mainly responsible for carbon tetrachloride metabolism and contributes to centrilobular necrosis similar to acetaminophen toxicity.

adenine nucleotides are other targets (Dahm and Jones, 1996; Sturgill and Lambert, 1997). The role of calcium and Kupffer cell activation have been implicated as contributing factors for acetaminophen-induced liver injury through the production of reactive nitrogen species (Treinen-Moslen, 2001).

Another classic example of xenobiotic bioactivation, generation of free radicals, leading to peroxidation, is conversion of carbon tetrachloride (CCl<sub>4</sub>) into trichlorocarbon radical (•CCl<sub>3</sub>) and then to trichloroperoxy radical (CCl<sub>3</sub>OO•) (Figure 17.5). The main phase I enzyme involved in bioactivation of both aforementioned acetaminophen and CCl<sub>4</sub> is CYP2E1, which is induced by alcohol consumption. Lipid peroxidation caused by free radicals increases the Ca<sup>2+</sup> permeability of the plasma membrane and leads to disruption of calcium homeostasis and centrilobular cell necrosis. In addition, •CCl<sub>3</sub> can directly bind to tissue macromolecules and lipid peroxy radicals can also form protein adducts (Jaeschke, 2008).

### Disruption of calcium homeostasis and cell membrane damage

Calcium ions (Ca<sup>2+</sup>) are important for the mediation of hepatocellular injury. Cytosolic-free calcium is maintained at relatively low concentrations compared to the extracellular concentrations. The majority of intracellular calcium is sequestered within the mitochondria and endoplasmic reticulum. Membrane-associated calcium and magnesium ATPases are responsible for maintaining this calcium gradient (Farrell *et al.*, 1990).

Disruption or changes in the permeability of the plasma membrane, mitochondrial membranes and

membranes of the smooth endoplasmic reticulum can lead to significant and persistent increases in the intracellular calcium. Depletion of available NADPH leads to calcium release, since calcium pumps in the mitochondrial membrane require NADPH (Cullen, 2005).

Excessive cytoplasmic calcium ions activate a variety of enzymes with further membrane damaging effects, including ATPases, phospholipases, proteases and endonucleases. Thus increased calcium causes increased mitochondrial membrane permeability and induction of apoptosis and necrosis. Calcium is required for cytoskeletal maintenance and function as well (Dahm and Jones, 1996; Delgado-Coello *et al.*, 2006). Increased calcium ions may also stimulate release of cytokines and eicosanoids by the Kupffer cells. Chemicals that cause liver damage by this mechanism include carbon tetrachloride, quinines, peroxides, acetaminophen, iron and cadmium.

### Mitochondrial injury

Mitochondria function in the production of energy, in the form of ATP, for cellular function by the process of oxidative phosphorylation. Hepatocytes are highly metabolically active and thus require a continuous supply of ATP. Hepatocytes active in detoxification or replication and replacement of damaged tissue have a still higher ATP requirement (Dahm and Jones, 1996). Compounds that disrupt mitochondrial oxidative phosphorylation include bile acids and amiodarone. Mitochondria are critical to modulation of cell redox status, osmotic regulation, pH control, cytosolic calcium homeostasis and cell signaling, and mitochondrial DNA is more susceptible to oxidant damage than nuclear DNA (Stirnemann *et al.*, 2010). Mitochondria are targets for virtually all types of injurious stimuli, including hypoxia and toxins including oxidants, electrophiles, lipophilic cations and weak acids. Damage is often precipitated by increases in cytosolic calcium.

Hepatic injury is frequently accompanied by morphological mitochondrial changes. These structural abnormalities include greatly increased size and the development of crystalline inclusions and are regarded as pathologic, reflecting either protective or degenerative responses to injury. Mitochondrial damage results in formation of high conductance channels, the so-called mitochondrial permeability transition, in the inner mitochondrial membrane. This is an irreversible change and, because membrane potential is critical for mitochondrial oxidative phosphorylation, constitutes a deathblow to the cell.

Oxidative phosphorylation produces reactive oxygen species which are deactivated within the mitochondria by antioxidants (Watkins, 1999). Glutathione within mitochondria functions as a scavenger for peroxides and

electrophiles. Synthesis of glutathione takes place within the cytosol and requires ATP. Greater than 90% depletion in the glutathione reserve decreases the ability of the mitochondrion to detoxify reactive oxygen species associated with ATP production (Watkins, 1999). Glutathione S-transferase, the enzyme required for recycling of glutathione, is sometimes overwhelmed by xenobiotics and reactive metabolites (Dahm and Jones, 1996).

Xenobiotics can cause cell death by their effects on mitochondrial DNA. Some antiviral deoxynucleoside analogs disrupt mitochondrial DNA synthesis through the inhibition of DNA polymerase gamma, thus depleting mitochondria, resulting in hepatocyte death.

Chemicals that damage mitochondrial structure, enzymes or DNA synthesis can disrupt beta oxidation of lipids and oxidative energy production within hepatocytes, which, if prolonged, leads to microvesicular steatosis which can progress to macrovesicular steatosis. This sequence of events is seen in alcoholic and non-alcoholic steatohepatitis. The role of mitochondria has been extensively studied with non-alcoholic fatty liver disease in people. Alcoholic steatosis and other forms of hepatic steatosis have been linked to impairment of ATP homeostasis and mitochondrial abnormalities have been reported in a growing body of literature. Aspirin, valproic acid and tetracyclines are also known to inhibit beta oxidation of fatty acids in mitochondria leading to lipid accumulation.

### Disruption of cytoskeleton

Changes in intracellular calcium homeostasis produced by active metabolites of xenobiotics may cause disruption of the dynamic cytoskeleton. There are a few toxins that cause disruption of the cytoskeleton through mechanisms independent of biotransformation. Microcystin is one of these toxins. Microcystin is produced by the cyanobacterium *Microcystis aeruginosa*. Similar toxins are produced by other species of cyanobacteria. The hepatocyte is the specific target of microcystin, which enters the cell through a bile-acid transporter. Microcystin covalently binds to serine/threonine protein phosphatase, leading to the hyperphosphorylation of cytoskeletal proteins and deformation of the cytoskeleton (Treinen-Moslen, 2001).

Phalloidin and related toxins found in some mushrooms, including *Amanita phalloides*, act by binding tightly to actin filaments and preventing cytoskeletal disassembly (Treinen-Moslen, 2001).

### Cholestasis

Cholestasis can be transient or chronic (Treinen-Moslen, 2001). If severe, bile pigments make the liver

appear grossly yellow to yellow-green (Plumlee, 2004). Cholestasis is subdivided into canalicular cholestasis and cholangiodestructive cholestasis.

Canalicular cholestasis can be produced by drugs/chemicals that damage the structure and function of bile canaliculi. A key component of bile secretion involves several ATP-dependent export pumps, such as the canalicular bile salt transporter that moves bile salts, and other transporters that export bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. Some drugs bind these transporter molecules, arresting bile formation and movement within the canalicular lumen (Klaassen and Slitt, 2005). Secondary bile injury results if there is cholestasis due to the detergent action of bile salts on the biliary epithelium or hepatocytes in areas of cholestasis.

Cholestasis can also occur simply as a result of physical obstruction of canaliculi within the liver parenchyma (intrahepatic) or outside the liver (extrahepatic). Causes of cholestasis include hepatobiliary tumors, endotoxemia, hepatocyte swelling and intraductal crystals such as calcium salts of plant saponins, for example those found in *Tribulus* spp. Most chemicals that cause cholestasis are excreted in the bile, including the mycotoxin sporidesmin, which concentrates 100-fold in the bile (Treinen-Moslen, 2001).

Disruption of actin filaments within the hepatocyte causes cholestasis by preventing the normal pulsatile contractions that move bile through the canalicular system to the bile ducts. Drugs that bind to actin filaments, such as phalloidin, those that affect cytoskeletal assembly, such as microcystin, and those that affect calcium homeostasis and cellular energy production generate this type of injury. Cholestatic disorders typically result in elevated serum alkaline phosphatase, gamma glutamyltransferase and serum bilirubin.

Cholangiodestructive cholestasis is caused by intrahepatic or extrahepatic bile duct obstruction. Injury of biliary epithelium leads to cell edema, sloughing into the lumen and inflammation, contributing to obstruction (Treinen-Moslen, 2001; Plumlee, 2004). Chronic lesions of cholangiodestructive cholestasis typically include bile duct proliferation and periductular fibrosis. Vanishing bile duct syndrome, characterized by a loss of bile ducts, has been described in chronic cholestatic disease in humans (Zimmerman, 1999; Treinen-Moslen, 2001) and produced experimentally in dogs (Uchida *et al.*, 1989).

## Hepatogenous photosensitization

Cholestatic diseases in herbivores, ruminants in particular, are associated with dermal photosensitization. The presentation of photosensitization is similar to that of sunburn, but with a more rapid onset and associated

with different wavelengths of light (Rowe, 1989). Photosensitization is caused by circulation of photoactive compounds. Primary photosensitization involves ingestion or dermal absorption of a photodynamic compound which enters the circulation, such as hypericin from *Hypericum perforatum* or St. John's wort, and is described elsewhere in this book. The second type of photosensitization is hepatogenous photosensitization, which is discussed in detail in this chapter.

Hepatogenous photosensitization usually occurs secondary to cholestasis. Herbivores ingest large quantities of chlorophyll. Metabolism of chlorophyll by bacteria in the gastrointestinal tract produces phylloerythrin, a photoactive compound, which, after absorption, is predominantly excreted in the bile (Rowe, 1989; Burrows and Tyrl, 2001). Cholestasis will prevent excretion of this photoactive compound. Upon exposure to light of wavelengths 320 to 400nm, circulating phylloerythrin contributes to generation of singlet oxygen, causing lipid peroxidation in areas of skin unprotected by hair or melanin (Burrows and Tyrl, 2001). Not all causes of cholestasis produce photosensitization, thus icterus may occur in the absence of photosensitization (Rowe, 1989).

Hepatocyte swelling may produce cholestasis. Agents that may cause photosensitization through hepatocyte damage include the toxic plant *Lantana camara* which causes steatosis. Plants containing pyrrolizidine alkaloids (such as *Senecio*, *Crotalaria*, *Cynoglossum* spp., *Tetradymia* spp., *Trifolium hybridum*, *Artemisia nigra*) also causes secondary photosensitization. Because significant hepatocyte damage must occur before the individual presents with photosensitization, and damage of this type usually carries a poor prognosis (Rowe, 1989).

Agents that damage bile ducts include the mycotoxin sporidesmin and saponogenins from the plant *Tribulusterrestris*. Sporidesmin, produced by *Pithomyces chartarum* which grows predominantly on ryegrass pastures in Australia and New Zealand, acts by directly damaging biliary epithelium, producing cell necrosis and degeneration. Bile ducts become occluded with cell debris and inspissated bile. Consequent periductular fibrosis also further occludes bile ducts (Rowe, 1989).

Several plant species cause bile stasis through precipitation of calcium salts of sapogenic glucuronides within bile ducts. The major toxic saponogenins from *T. terrestris* are diosgenin and yamogenin. These compounds are hydrolyzed in the gastrointestinal tract to sapogenins, which are further metabolized before glucuronidation in the liver (Burrows and Tyrl, 2001). Sapogenins directly occlude bile ducts and damage canalicular membranes. Note that while *T. terrestris* poisoning is reported with some frequency in South Africa and Australia, it is believed that the toxic sapogenins are either absent or present in much lower concentrations in *T. terrestris* growing in the United States (Burrows and Tyrl, 2001).



## Inhibition of tissue repair response

Tissue repair is a dynamic response, involving compensatory cell proliferation and tissue regeneration, which is stimulated in acute toxicosis to recover hepatic structure and function. Extensive evidence from rodent models using structurally and mechanistically diverse hepatotoxins such as acetaminophen, carbon tetrachloride, chloroform, thioacetamide, trichloroethylene and allyl alcohol have demonstrated that tissue repair plays a critical role in determining the final outcome of toxic insult, i.e., recovery from injury and survival or progression of injury leading to liver failure and death (Mehendale, 2005).

Thioacetamide, originally used as a fungicide, is potentially hepatotoxic after being bioactivated by cytochrome P450 and/or flavin-containing monooxygenase (FMO) systems to sulfinic (sulfoxide) and sulfenic (sulfone) metabolites, which cause centrilobular necrosis. Thioacetamide bioactivation is primarily mediated by the cytochrome P450 enzyme CYP2E1. Studies suggest that thioacetamidesulfoxide, a relatively stable intermediate of thioacetamide metabolism, is obligatory for the hepatotoxic effects, indicating that it is the penultimate reactive metabolite. Accordingly, it has been reported that the hepatotoxic effects of thioacetamide are only expressed after metabolic conversion to thioacetamide S-oxide, which undergoes further metabolism to an as yet unidentified metabolite, most likely the reactive unstable thioacetamidesulfone. The reactive metabolite of thioacetamide binds to liver proteins with the formation of acetylimidolysine derivatives that are responsible for thioacetamide-induced hepatotoxic effects. Progression of thioacetamide-induced liver injury has also been attributed to inhibition of tissue repair response based on studies of diet restriction (Ramaiah and Mehendale, 2000).

Tissue repair is a complex process governed by intricate cellular signaling involving chemokines, cytokines, growth factors and nuclear receptors, leading to promitogenic gene expression and cell division. Tissue repair encompasses regeneration of hepatocytes, hepatic extracellular matrix and angiogenesis, processes necessary to completely restore hepatic structure and function after injury. New insights have emerged over the last quarter century indicating that tissue repair follows a dose response: increasing xenobiotic dose, a threshold is reached beyond which repair is delayed or impaired through inhibition of cellular signaling. Runaway secondary events then cause tissue destruction, organ failure and death. Prompt and adequate tissue repair after toxic injury is critical for recovery. Tissue repair is modulated by species, strain, age, nutrition, disease condition and other factors, accounting for the marked differences in individual susceptibility and toxic outcome (Ramaiah *et al.*, 1998; Mehendale, 2005).

## IDIOSYNCRATIC REACTIONS

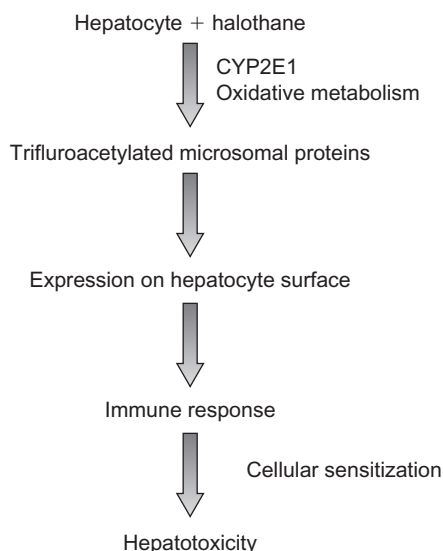
Idiosyncratic drug reactions often occur after sensitization followed by reexposure to a drug and are classified as immune-mediated idiosyncratic hepatotoxicity (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Zimmerman, 1999; Treinen-Moslen, 2001). A delay of 3 to 4 weeks after 1 to 2 week course of medication can be seen for these drugs (e.g., amoxicillin-clavulanic acid) before clinical signs become evident (Kaplowitz, 2001; Zimmerman, 1999), but onset is expedited with rechallenge (Dahm and Jones, 1996; Sturgill and Lambert, 1997; Watkins, 1999).

Neoantigens may result from adducts formed by interaction of reactive drug metabolites with cellular proteins. These neoantigens could be processed by Kupffer cells or other antigen presenting cells, transported to the cell surface and presented to the immune system as antigens. Cell and antibody-mediated immune response can cause severe liver damage. Various drugs are believed to cause immune-mediated idiosyncratic reactions in humans, including halothane, diclofenac, phenytoin and sulfonamides (Sturgill and Lambert, 1997; Watkins, 1999; Zimmerman, 1999; Treinen-Moslen, 2001). The idiosyncratic reaction to halothane has been well studied (Figure 17.6).

Other idiosyncratic drug reactions can be seen after a very long latency (up to 12 months), but are usually not associated with features of hypersensitivity and have been variable in response to a rechallenge. These are classified as nonimmuno-mediated idiosyncratic reactions (Kaplowitz, 2001). Examples of drugs that are known to cause this type of idiosyncratic reaction include troglitazone, valproate, amiodarone, ketoconazole, disulfiram and isoniazid. However, some involvement of allergic mechanism cannot be completely rejected in these drugs, thus this classification should still be viewed as tentative (Kaplowitz, 2005).

Many drugs implicated in idiosyncratic liver injury are known to affect mitochondrial function (Stirnemann *et al.*, 2010). A proposed explanation for the delayed onset of clinical signs is that mitochondrial damage is not manifested until a threshold has been reached, then cell death occurs. Genetic polymorphisms in activation of detoxifying enzymes have been associated with increased susceptibility to idiosyncratic drug reactions in some people, further implicating the role of oxidative stress (Stirnemann *et al.*, 2010).

A few idiosyncratic drug reactions have been reported in veterinary medicine. The most well known is probably carprofen in dogs (MacPhail *et al.*, 1998). Carprofen is a nonsteroidal anti-inflammatory agent. Clinical signs of the idiosyncratic reaction to carprofen occur approximately 20 days following the first exposure to the drug and include anorexia, vomiting and icterus. Signs do not correlate with drug dose. Affected dogs have elevated



**FIGURE 17.6** Postulated mechanism of immune-mediated halothane hepatotoxicity. Halothane-mediated hepatitis is the best example for immune-mediated liver damage. Oxidative pathway yields trifluoroacetylchloride, which can react with microsomal proteins to form a neoantigen which then can generate immune response leading to hepatic injury.

alanine transaminase, aspartate transaminase, alkaline phosphates and serum total bilirubin. There is mild to severe bridging hepatocyte degeneration and necrosis with evidence of apoptosis. Mild to moderate periportal inflammation is reported. Spayed female dogs are over-represented among the affected (MacPhail *et al.*, 1998).

Other idiosyncratic reactions of veterinary importance include diazepam in cats (Center *et al.*, 1996) and sulfonamides in dogs (Trepanier *et al.*, 2003; Shenton *et al.*, 2004; Trepanier, 2004). The clinical presentation associated with diazepam in cats is similar to that described with carprofen in dogs. Idiosyncratic reactions to sulfonamides occur in about 0.25% of dogs and a variety of organ systems may be affected, hepatopathy being the third most reported sequela. Other effects may include polyarthropathy, which predominates in Doberman pinschers, thrombocytopenia, pyrexia and dermal drug eruptions.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Research in the last decade has focused on understanding the different mechanisms for chemical-induced liver injury. Researchers have attempted to understand the molecular basis for injury and the contribution of individual cell types to ultimate hepatic pathology. Recently,

techniques such as microarray-based toxicogenomics, 2D gel electrophoresis, mass spectrometry-based proteomics and  $^1\text{H}$ -NMR spectroscopy-based metabolomics have attempted to further design novel hypotheses to test the molecular players involved in liver damage (Craig *et al.*, 2006; Blomme *et al.*, 2009; Kienhuis *et al.*, 2011).

Toxicogenomics will enable the detection of specific biomarkers used to predict adverse drug effects at low-level exposure (Kienhuis *et al.*, 2011). Several signature biomarkers have been elucidated that may eventually be useful in the diagnosis of hepatotoxicosis: serum proteomic analysis revealed increased concentrations of vitamin D-binding protein, malate dehydrogenase and purine nucleoside phosphorylase in several models of chemically induced hepatopathies. These enzymes were elevated in the serum before increases in conventional serum markers such as transaminase, alkaline phosphates or microscopic changes (Amacher *et al.*, 2005). Once validated for use in humans and domestic animals, these proteins are potentially predictive of hepatotoxicity. Use of these biomarkers will assist in identifying drugs that can cause serious adverse reactions, such as idiosyncratic liver injury, which cannot be easily discovered during conventional pre-clinical or clinical drug trials. Further, research involving transgenic and knockout models provides insight into the mechanistic and molecular basis of liver injury.

Genetic predispositions to idiosyncratic drug reactions have been elucidated in humans, such as sensitivity to diclofenac and other drugs and xenobiotics in patients with a mutation in the gene for multidrug resistance-associated protein 2. Mutations in the genes for superoxide dismutase and glutathione transferase also account for increased susceptibility to the toxic effects of certain drugs (Stirnemann *et al.*, 2010). Animal models for idiosyncratic drug reactions remain elusive, but form another area requiring investigation.

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## Renal toxicity

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### INTRODUCTION

The kidney plays a vital role in maintaining homeostasis through the elimination of waste products, conservation of fluid and electrolyte balance, maintenance of acid–base balance, secretion of hormones and regulatory peptides, and metabolism and excretion of both endogenous compounds and xenobiotics. Although the kidney comprises roughly 0.5% of bodily mass, it receives up to 20–25% of cardiac output which can expose renal tissue to relatively high levels of blood-borne toxicants. The kidney may also be exposed to toxicants through the metabolic activation of xenobiotics by enzyme systems within the renal tubular epithelium, and through the process of urine concentration, the kidney can be exposed to higher concentrations of toxicants than other tissues. Injury to the kidney can lead to widespread systemic derangements, and due to limited regenerative ability, long-term renal insufficiency may result from exposure to nephrotoxic agents.

### FUNCTIONAL ANATOMY

Kidneys are paired organs residing ventrolaterally to the lumbar vertebrae in mammals. Mammalian kidneys are bean to horseshoe shaped with uniform exterior surfaces, although some species (e.g., bears, ox, whales) have multilobulated surfaces. Kidneys can be unipyramidal (e.g., horses, dogs, cats) or multipyramidal (e.g., pigs, ox) depending on the number of renal papillae into which renal lobes empty. Mammalian kidneys

are of equal size and are roughly the equivalent of three vertebrae in length. The surface of the kidney is covered by a fibrous capsule, and is brown-red in color in most species; in the cat, normal high fat content within the tubules results in a kidney that is a pale, yellow-gray color. Viewed on sagittal section, the kidneys have a medial indented hilar region from which renal artery, renal vein, lymphatics, nerves and ureter emerge. Viewing the cut surface of the sagittally incised kidney, the renal interior can be divided into two distinct sections, the cortex and medulla. The renal cortex in mammals comprises approximately 80% of the renal mass, and the normal cortex to medulla ratio is 1:2 to 1:3 in most species (Maxie and Newman, 2007). The medulla is divided into ray-shaped sections known as renal pyramids, which have their bases at the corticomedullary junction and apices that empty into the renal calyx (cats, ox) or pelvis (dogs, horses). From the hilar region, the ureter directs urine to the distal urinary bladder.

The functional unit of the kidney is the nephron, which is comprised of the renal corpuscle (Bowman's capsule and the glomerulus), proximal tubule, loop of Henle and distal tubule. The number of nephrons per kidney ranges from 200,000 in cats and 400,000 in dogs to 1,000,000 in humans. The ultimate number of nephrons is fixed at birth, although kidneys of altricial offspring of some species (e.g., dog, cat, pig) undergo nephrogenesis for several weeks after birth (Maxie and Newman, 2007). Renal blood flow originates from the renal arteries which are direct branches from the aorta. The kidneys receive 20–25% of cardiac output, allowing the entire plasma volume to be filtered approximately 100 times daily. Renal arteries progressively branch to form interlobar arteries, arcuate arteries, interlobular

arteries and afferent arterioles, which feed blood to the glomerulus. The high hydrostatic pressure from afferent arterioles provides the force for ultrafiltration of plasma by the glomerulus, a tuft of branching and anastomosing capillaries. The glomerular “sieve” consists of the capillary endothelium, basement membrane and epithelial *podocytes* that anchor foot processes (*pedicels*) within the lamina rara of the glomerular basement membrane (GBM). These pedicels are separated by filtration slits covered by slit diaphragms containing 6–9 nanometer diameter pores through which plasma is filtered, permitting filtration of compounds up to approximately 60 kilodaltons in size. The glomerular mesangium provides the supporting framework for the glomerular capillaries, and is composed of an extracellular matrix, contractile stellate cells which respond to vasoactive hormones and phagocytic cells which are involved in local immune reactions (Khan and Alden, 2002). The distal ends of glomerular capillaries merge to form efferent arterioles that progress further down the nephron and form the peritubular capillary network that surrounds the renal tubules and helps to drive the countercurrent absorption mechanisms responsible for concentration of the urine and regulation of electrolyte balance. The majority of renal blood flow and oxygen is expended in the cortex, making the medulla a relatively hypoxic area of the kidney.

Fluid filtered by the glomerulus enters Bowman’s capsule and flows into the proximal renal tubule. The proximal tubule is divided into three segments that differ anatomically and functionally. The most proximal segment,  $S_1$ , consists of the convoluted portion of the proximal tubule and possesses epithelial cells with tall brush borders, well-developed lysosome systems and numerous basally located mitochondria. The  $S_2$  segment extends from the end of the convoluted segment to the beginning of the straight segment and constitutes a transition segment.  $S_2$  segment epithelia have shorter brush borders, fewer mitochondria and fewer lysosomes than cells in the  $S_1$  segment. The  $S_3$  segment encompasses the remaining distal portion of the proximal tubule and extends into the outer reaches of the medulla. Oxygen consumption,  $\text{Na}^+/\text{K}^+$  ATPase activity and gluconeogenic capacity are greatest in the first two segments, while the  $S_3$  segment has higher transport capabilities for some compounds, such as ascorbic acid (Castro *et al.*, 2008). The  $S_3$  segment is also the site for metabolic activation of some toxicants.

In the proximal tubule, passive reabsorption of water and active reabsorption (via  $\text{Na}^+/\text{K}^+$  ATPases) of sodium occurs. Other solutes reabsorbed by the proximal tubule include potassium, calcium, phosphorus, bicarbonate, glucose, amino acids and proteins, and various xenobiotics, with each segment having a different range of and capacity for reabsorption. For instance, the  $S_1$  segment

is primarily responsible for the reabsorption of bicarbonate, glucose, amino acids and low molecular weight proteins. The proximal tubule ultimately reabsorbs 60–80% of solute and water filtered by the glomerulus. Excretory functions of the proximal tubule include the active secretion of weak organic anions and cations. The proximal tubular epithelium possesses cytochrome P450-dependent mixed function oxidases capable of metabolizing a variety of endogenous compounds and xenobiotics. Agents that are biotransformed to toxic metabolites (e.g., acetaminophen) can induce proximal renal tubular injury.

Filtrate passes from the proximal tubule into the loop of Henle, which has a thin-walled descending limb and a thick-walled ascending limb that extend to the level of the outer medullary region. In some nephrons, termed long-looped nephrons, the loop dips deeply into the inner medulla; in these nephrons, the proximal aspect of the ascending limb is thin-walled, becoming thick-walled as the tubule reaches the level of the outer medulla. Countercurrent exchange mechanisms within the loop result in the reabsorption of approximately 20% of filtered water and 25% of filtered sodium and potassium (Schnellmann, 2008). In the thin descending limb, water and interstitial solutes such as urea and electrolytes are able to freely pass into the tubule. At the level of the thin ascending limb, the tubule becomes impermeable to water and urea, and chloride and sodium ions are actively transported via  $\text{Na}^+/\text{K}^+$  ATPases. Because the loop of Henle resides largely within the poorly oxygenated renal medulla, the high oxygen demand of the  $\text{Na}^+/\text{K}^+$  ATPases make this segment especially susceptible to hypoxic injury (Brezis *et al.*, 1984).

The macula densa is composed of specialized cells located between the end of the thick ascending limb of the loop of Henle and the most proximal aspect of the distal tubule. This area is in close proximity to afferent arterioles, allowing for communication between the macula densa and afferent arteriole of the same nephron. An increase in intratubular solute concentration at the macula densa results in a feedback signal to the afferent arteriole, resulting in vasoconstriction and reduction of glomerular filtration rate (GFR). This feedback mechanism helps prevent massive loss of fluid and solute in the face of impaired renal tubular absorption. The proximal-most aspect of the renal distal tubule is responsible for reabsorption of most of the remaining intraluminal electrolytes such as sodium and potassium. The remaining segment of the distal tubule and collecting duct absorb remaining sodium, eliminate excessive potassium and hydrogen ions, and absorb additional water (influenced by antidiuretic hormone) as needed to regulate the volume and composition of the urine. Agents that disrupt the osmotic gradient of the medullary region (e.g., by increasing medullary blood flow) can disrupt

the ability of the kidney to concentrate urine. Collecting ducts progressively intersect and anastomose toward the renal papilla and ultimately empty into the renal calyx, renal pelvis, or ureter, depending on species. Peristaltic action of the ureter propels urine toward the urinary bladder for temporary storage and elimination.

In addition to its role in regulation of waste excretion and water/electrolyte balance, the kidney secretes a variety of hormones and regulatory peptides vital for normal systemic homeostasis. Secretion of erythropoietin by renal peritubular interstitial cells promotes red blood cell formation; significant chronic renal disease is often associated with anemia due to decreases in erythropoietin secretion. Renin secreted from the juxtaglomerular cells increases systemic and renal blood pressure and aldosterone release. Prostaglandins and prostacyclin are produced by a variety of renal cells and aid in regulation of renal vascular tone, mesangial contractility and processing of water and electrolytes by the renal tubules. The kidney also plays an important role in the xenobiotic clearance and/or metabolism.

## TOXIC EFFECTS ON THE KIDNEY

### Acute renal failure

Acute renal failure is defined as an abrupt decrease in renal function leading to retention of nitrogenous wastes (Langston, 2010), and is one of the most common manifestations of nephrotoxic injury (Schnellmann, 2008). The term *acute renal failure* is sometimes used interchangeably with *acute renal insufficiency* or *acute renal injury* (AKI). The latter term has been suggested as the proper term to use in order to encompass the entire spectrum of renal injury, from minor elevations of serum chemistry values (i.e., blood urea nitrogen and creatinine) to anuric renal failure.

The primary manifestation of AKI is a decrease in GFR leading to an excess of nitrogenous wastes in the blood (azotemia). Decreases in GFR may result from prerenal, renal or postrenal causes. Prerenal causes of decreased GFR include hypovolemia, renal vasoconstriction and poor cardiac output. Postrenal factors include obstruction of the ureters, bladder or urethra (e.g., bladder stones). Primary renal factors that can result in AKI leading to decreased GFR include tubular injury, glomerular injury, interstitial disease and renal vascular compromise. In humans, prerenal factors are said to account from 20 to 80% of cases of AKI, while renal factors account for 10 to 45% and postrenal factors account for 5 to 15% (Langston, 2010). Of causes of primary renal injury, ischemia/reperfusion and nephrotoxicosis are considered to account for over 90% of AKI cases (Schnellmann,

2008). Nephrotoxics damage the kidneys by a variety of different mechanisms including: (1) direct injury to renal tubular epithelium, leading to epithelial cell necrosis, sloughing and obstruction of tubules by cellular debris (tubular casts), (2) detachment of lethally injured cells from the basement membrane, resulting in back-leakage of filtrate across the exposed basement membrane and adherence of detached cells to sub-lethally injured cells still attached to the basement membrane causing lumen obstruction, (3) renal vasoconstriction, resulting in hypoxia and ischemic necrosis of renal structures, (4) damage to the glomerular filtration barrier, and (5) impairment of renal healing and repair (Counts *et al.*, 1995). Most nephrotoxics exert their damage at the level of the renal tubules, with many toxicants targeting specific segments of the tubules. For instance, aminoglycoside antibiotics cause damage primarily to the S<sub>1</sub> and S<sub>2</sub> segments of the proximal tubule, while mercuric chloride-induced injury is restricted to the S<sub>3</sub> segment at low doses, extending into the S<sub>2</sub> and S<sub>1</sub> segments only at higher doses (Diamond and Zalups, 1998).

The clinical signs of acute renal injury and/or failure can include polydipsia, nausea or vomiting, lethargy, anorexia, weakness, dehydration and polyuria/oliguria/anuria. More severe cases may have halitosis, oral ulceration, abdominal (renal) pain, palpably enlarged kidneys and cardiac arrhythmias. Clinical laboratory abnormalities indicative of AKI include elevations in blood urea nitrogen and serum creatinine (azotemia), hyperphosphatemia, hyper- or hypokalemia and metabolic acidosis. *Uremia* is the term used when azotemia is accompanied by typical clinical signs of AKI. Advanced cases of uremia may present with gastrointestinal ulceration, anemia, peripheral neuropathy, encephalopathy and cardiac dysfunction.

The ability of the kidney to heal following an acute toxic insult is dependent upon several factors including the dose and type of toxicant, the amount of functional kidney remaining, the presence and severity of secondary uremic conditions (e.g., soft tissue mineralization) and the degree of medical intervention and supportive care provided during the acute crisis. Mild to moderate renal tubular injury with retention of tubular basement membranes have a reasonable prognosis for tubular regeneration provided that supportive care is administered until tubules have had a chance to recover. In uncomplicated acute tubular injuries, regeneration of epithelial cells generally begins after about 7–10 days following the renal insult; in mild cases, full recovery of architecture may occur within 2–3 weeks, with longer recovery periods being required for more severe renal injury (Maxie and Newman, 2007). In situations where nephrons have been fully obstructed by cellular debris or crystals, or if basement membrane integrity is lost, regeneration may be incomplete, resulting in

long-term renal insufficiency and/or progression to chronic renal failure.

## Chronic renal failure

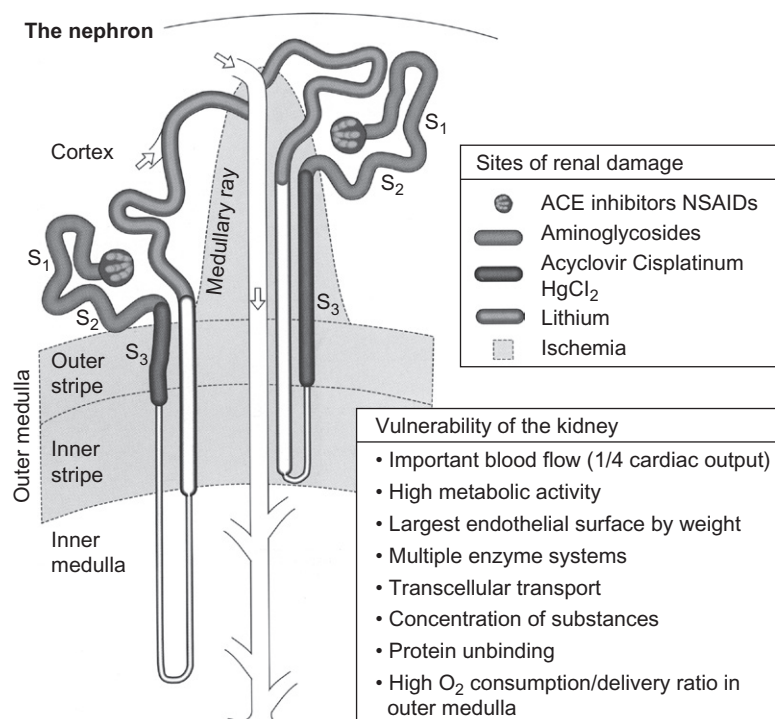
Chronic renal failure (CRF) is most commonly the result of long-term exposure to toxicants, and many of the alterations seen in CRF are related to the secondary compensatory changes triggered by the initial injury. Upon loss of nephrons (and thus decrease in overall renal GFR), hemodynamic alterations occur that increase the blood flow and pressure to surviving nephrons in an attempt to reestablish normal whole-kidney GFR. Although changes such as increased glomerular pressures can help to maintain overall GFR, these increased pressures may contribute to glomerular sclerosis, tubular atrophy and interstitial fibrosis, thus furthering the progression of renal injury (Brenner *et al.*, 1982). Increased pressure within glomerular capillaries results in an increase in volume of the glomerular tuft, glomerular hypertrophy and intraglomerular hypertension. Consequences of these changes include hyaline accumulation within glomerular capillary walls, mesangial dysfunction associated with matrix accumulation and microaneurysm formation, and thrombosis due to endothelial injury (Polzin, 2010). As the glomerulus expands, podocytes are unable to maintain the integrity of slit diaphragms and focal denudation of GBM occurs, allowing leakage of larger proteins into the glomerular filtrate (proteinuria). In addition to hemodynamically induced progression of CRF, there is

evidence to suggest that T-lymphocytes and interleukin-6 contribute to the ongoing interstitial fibrosis in dogs with chronic progressive kidney disease (Yhee *et al.*, 2008).

Clinical effects associated with CRF include uremia, gastrointestinal disorders (uremic gastritis, uremic enterocolitis), polyuria, polydipsia, nocturia, dehydration, atrial hypertension, peripheral neuropathy, uremic encephalopathy, myopathy, anemia, platelet dysfunction, renal secondary hyperparathyroidism, cachexia and hypokalemia (especially in cats) (Polzin, 2010). Immunosuppression is a potential complication of CRF in humans, and studies of dogs with CRF have shown impaired immunological function as well (Kravola *et al.*, 2010). Animals with CRF may show few outward signs of illness until late in the course of their disease; in some instances acute decompensation may occur during periods of physical or emotional stress (Hosseini and Hosseini, 2008). Lesions found in animals with CRF include kidneys that are small and irregular in shape, with uneven capsular surfaces. On cut section, pale streaks (fibrosis) may be seen within the interstitium and the parenchyma may be gritty upon cutting due to mineralization and/or crystal deposition.

## Patterns of toxic renal injury

Identification of the target site of action of nephrotoxins can assist in determining the functional impact and potential mechanism of toxicity (Figure 18.1). Toxicants that are directly toxic to cells may cause injury to the glomerulus or the S<sub>1</sub> segment of the proximal tubule as



**FIGURE 18.1** Sites of renal damage, including factors that contribute to the kidney's susceptibility to damage. ACE: angiotensin converting enzyme; NSAIDs: nonsteroidal anti-inflammatory drugs; HgCl<sub>2</sub>: mercuric chloride. (Adapted from Berl T and Bonaventura JV (1998) *Atlas of Diseases of Kidney*, Schrier RW (ed.). Blackwell Publishing, Philadelphia, with permission.)



they first enter the nephron. Conversely, direct-acting toxicants may be dilute in the initial ultrafiltrate, and may not cause injury until they reach the more distant nephron, where they may reach toxic concentrations as water is gradually reabsorbed and/or the pH changes.

Some toxicants require bioactivation in order to exert their toxic effects, so they may cause site-specific injury to the S<sub>3</sub> segment of the proximal tubule, where bioactivation processes are most active. A list of nephrotoxicants and their primary sites of action can be seen in Table 18.1.

TABLE 18.1 Urinary tract toxicants listed by site of injury

Toxicant	Species	Lesion	Comment
<b>Glomerular injury</b>			
Bacterial endotoxin (hemolytic-uremia syndrome)	All	Mesangiolysis	Intravascular fibrin deposition of glomerular capillaries (Schwartzman reaction)
Crotalid snake venom	All	Mesangiolysis	Also tubular degeneration
Cyclosporine	Dogs, cats	Thrombotic microangiopathy	
Gold salts	Dogs	Membranous glomerulonephritis	Also acute tubular necrosis, vasculopathy, interstitial fibrosis
Mercury	Ox	Membranous glomerulonephritis; proximal tubule degeneration and necrosis	Affects S <sub>1</sub> segment of proximal tubule
<b>Proximal tubular injury</b>			
Acer rubrum (red maple)	Horses	Acute tubular degeneration and necrosis; hemoglobin casts in tubules	
Acetaminophen	All	Acute tubular degeneration and necrosis	Metabolic activation results in damage to S <sub>3</sub> ; hepatotoxicity more common
<i>Amaranthus</i> spp.	Herbivores, especially pigs, calves	Acute tubular degeneration and necrosis; perirenal edema	See text for more information
Aminoglycoside antibiotics	All	Acute tubular degeneration and necrosis	Predominantly S <sub>1</sub> and S <sub>2</sub> segments; see text for more information
Amphotericin B	Dogs, cats	Acute tubular degeneration and necrosis; tubular dilatation; glomerular and interstitial mineralization	Arteriolar vasoconstriction results in decrease in GFR
Arsenic	All	Acute tubular degeneration and necrosis, cortical necrosis (dogs)	All segments
Cadmium	All	Acute tubular degeneration and necrosis	Glomerular injury reported in seals <sup>a</sup>
Cantharadin (blister beetle, <i>Epicutata</i> spp.)	Herbivores, especially horses	Acute tubular degeneration and necrosis; renal cortical hemorrhages; hyperemia and hemorrhage of mucosa of renal crest, ureter, bladder, urethra	Vesicant effect on mucosa of alimentary tract, cardiac lesions
Carbon tetrachloride	All	Tubular degeneration and necrosis	Hepatic injury most prominent
Cephalosporin antibiotics (and other $\beta$ -lactam antibiotics)	All	Tubular degeneration and necrosis	High intracellular concentrations in S <sub>3</sub> segment due to action of organic ion transporter result in cell injury
Cholecalciferol (1,25-dihydroxy cholecalciferol, vitamin D <sub>3</sub> ), calcitriol, calcipotriene, <i>Cestrum diurnum</i> , <i>Solanum</i> spp., <i>Trisetum</i> spp.	All	Tubular degeneration and necrosis; mineralization	Renal ischemia due to vasoconstriction and mitochondrial calcification secondary to hypercalcemia <sup>b</sup>
Cisplatin	Dog	Tubular degeneration and necrosis	Primary effect on S <sub>3</sub> segment due to metabolic activation
Citrinin	All species, pigs most common	Tubular degeneration and necrosis	Primary effect on S <sub>2</sub> segment, thick ascending limb, distal convoluted tubule and collecting ducts; see text for more information
Copper	Sheep, other ruminants less common	Tubular degeneration and necrosis; hemoglobinuric and bile nephrosis (gunmetal kidney)	Non-ruminants develop liver injury without hemolysis
Ethylene glycol	All, dogs and cats most common	Tubular degeneration and necrosis; intratubular calcium oxalate crystals	Oxalate crystals may be found in vessels of meninges, heart, liver, intestinal mucosa; see text for more information

(Continued)

TABLE 18.1 (Continued)

Toxicant	Species	Lesion	Comment
<i>Lantana</i> spp.	All grazing animals except horses	Tubular degeneration and necrosis	Lesions primarily in liver; secondary photosensitization; cardiac lesions
Lead	All	Tubular degeneration and necrosis; intranuclear inclusion bodies; karyomegaly of tubular epithelium	
<i>Lilium</i> spp.	Cats only	Tubular degeneration and necrosis; vascular fibrinoid necrosis	See text for more information
NSAIDs (see distal tubule section for phenylbutazone and flunixinmeglumine)	Dogs, cats, vultures, other species likely susceptible	Tubular degeneration and necrosis; occasionally papillary necrosis in dogs	See text for more information
Ochratoxin A	All, pigs most commonly affected	Tubular degeneration and necrosis; cystic tubular dilatation; interstitial fibrosis; renal edema in chickens	Primary effect on S <sub>3</sub> segment due to bioactivation; <sup>c</sup> carcinogen; see text for more information
Oosporein	Chickens	Tubular degeneration and necrosis; interstitial pyogranulomatous inflammation and fibrosis	Periodic-acid-Schiff-positive granules in macula densa <sup>d</sup>
Oxalic acid, soluble oxalates ( <i>Halogetonglomeratus</i> , <i>Sarcobatusverniculatus</i> , <i>Rheum rhaponticum</i> , <i>Rumex</i> spp., <i>Chenopodium</i> spp.)	All, ruminants and pigs most common	Tubular degeneration and necrosis; intratubular calcium oxalate crystals	Precipitation of soluble oxalates with serum calcium; hypocalcemic tetany possible
Paraquat	All	Tubular degeneration and necrosis	Pulmonary lesions most common effect (progressive pulmonary fibrosis); corrosive lesions in GI tract
Pine oil	Cats	Tubular degeneration and necrosis	Hepatic lesions more prominent
Pyrrolizidine alkaloids ( <i>Amsinckia</i> , <i>Crotolaria</i> , <i>Cynoglossum</i> , <i>Echium</i> , <i>Heliotropium</i> , <i>Senecio</i> spp.)	All, predominantly horses, cattle, pigs	Tubular degeneration and necrosis; megalocytosis of tubular and glomerular cells	Hepatic lesions more prominent; secondary photosensitization, hepatic encephalopathy
Quercus spp. (Oaks)	Grazing animals, cattle most common	Tubular degeneration and necrosis; perirenal edema	See text for more information
Sulfonamide antibiotics	Ruminants	Tubular degeneration; grossly visible intratubular crystals	
<i>Vitis</i> spp. (grapes, raisins, Zante currants)	Dogs	Tubular degeneration and necrosis	See text for more information
Zinc	All	Tubular degeneration and necrosis; hemoglobinuricnephrosis	
<b>Distal tubular/collecting duct injury</b>			
Fluoride	All	Distal tubular degeneration and necrosis	
Melamine: cyanuric acid	Dogs, cats, raccoon dogs, pigs, rats, fish; others likely susceptible	Distal tubular degeneration and necrosis; crystals in distal tubules and collecting ducts	See text for more information
<b>Interstitial injury</b>			
<i>Vicia</i> spp. (vetch)	Grazing animals, primarily cattle	Eosinophilic granulomatous nephritis	Lesions can occur in any organ
<b>Lower urinary tract injury</b>			
Cyclophosphamide	Dogs	Hemorrhage and necrosis of urinary bladder	Bioactivation and concentration of toxic metabolites in urine
Ptaquiloside ( <i>Pteridium</i> spp., bracken fern)	Cattle	Necrosis and hemorrhage of urinary bladder; urinary bladder neoplasia	Enzootic hematuria
Ptaquiloside ( <i>Sorghum</i> spp.)	Horses	Necrosis and hemorrhage of urinary bladder	Equine cystitis ataxia syndrome

<sup>a</sup>Sonne-Hansen C, Dietz R, Liefsson PS, Hyldstrup L, Riget FF (2002) Cadmium toxicity to ringed seals (*Phoca hispida*): an epidemiological study of possible cadmium-induced nephropathy and osteodystrophy in ringed seals (*Phoca hispida*) from Qaanaaq in Northwest Greenland. *Sci Total Environ* 295: 167–181.

<sup>b</sup>Haschek WM, Rousseaux CG, Wallig MA (2007) Kidney and lower urinary tract. In *Fundamentals of Toxicologic Pathology*, 2nd edn. Academic Press, San Diego, pp. 221–238.

<sup>c</sup>Boorman GA, McDonald MR, Imoto S, Persing R (1992) Renal lesions induced by ochratoxin A exposure in the F344 rat. *Toxicol Pathol* 20: 236–245.

<sup>d</sup>Brown TP, Fletcher OJ, Osuna O, Wyatt RD (1987) Microscopic and ultrastructural renal pathology of oosporein-induced toxicosis in broiler chicks. *Avian Dis* 31: 868–877.

## Glomerular injury

The glomerular capillaries are the first component of the nephron to be exposed to blood-borne toxicants. The glomerular cells, matrix and mesangium are susceptible to toxic injury by several different mechanisms including direct injury to cellular components, formation of oxygen-derived free radicals, disruption of extracellular substrates (e.g., basement membranes), immune-mediated injury and disruption of renal hemodynamics (Khan and Alden, 2002). Direct injury or injury secondary to reactive oxygen intermediates can result in endothelial loss, glomerular podocyte injury and necrosis of mesangial cells and substrate (mesangiolysis). Further damage may occur secondary to cytokines released by inflammatory cells responding to the site of injury. Alterations in the GBM secondary to cellular injury can result in disruption of the glomerular filtration barrier, leading to proteinuria. Deposition of and immune-mediated reaction to various toxicants (e.g., mercurial, gold salts) can result in thickening of the GBM, disrupting the glomerular filtration barrier and leading to membranous glomerulonephropathy.

## Proximal tubular injury

Tubular injury is the most common pattern of renal injury induced by toxicants and the proximal tubule is most frequently affected by nephrotoxicants (Schnellmann, 2008). Damage to the proximal tubule may occur due to direct damage from toxicants, metabolic activation of toxicants, ischemia-reperfusion or physical or chemical disruption of endothelium and/or basement membrane. The S<sub>1</sub> segment is the most vulnerable to injury from toxicants that exert direct injury as the epithelium in this area is exposed to the toxicant first. The proximal convoluted tubule epithelium is actively involved in endocytosis of various compounds that bind to the brush border, sequestering the compounds in phagolysosomes. When this process is overwhelmed by the presence of certain toxicants (e.g., aminoglycoside antibiotics), loss of phagolysosome membrane integrity occurs, resulting in lysosomal leakage and cell injury or necrosis (Khan and Alden, 2002). This type of injury is most commonly associated with the S<sub>1</sub> and S<sub>2</sub> segments of the proximal tubule. In contrast, the straight segment (S<sub>3</sub>) is most susceptible to injury by metabolic activation, transporter-associated accumulation and ischemia reperfusion.

## Distal nephron/renal papillary injury

Toxic injury to the distal nephron is relatively uncommon, and injury to this area generally manifests as

decreased urine concentration ability or defects in acid secretion. Injury to the renal papilla is most commonly seen with chronic consumption of non-steroidal anti-inflammatory drugs (NSAIDs), although use of NSAIDs in hypovolemic animals or in conjunction with other nephrotoxicants (e.g., aminoglycoside antibiotics) has also been associated with the development of renal papillary necrosis (Talcott, 2006).

## NEPHROTOXIC AGENTS

### Mycotoxins

#### Ochratoxins

Ochratoxins are produced by various species of the fungi *Aspergillus* and *Penicillium* and are produced on a variety of cereal grains, cottonseed, nuts, dried beans and coffee beans (Haschek *et al.*, 2002). OchratoxinA (OTA) is the most common and most toxic of the ochratoxins that have been identified to date. The co-production of penicillic acid by *Penicillium ochraeus* or citrinin by *Aspergillus* spp. or *Penicillium* spp. greatly enhances the toxicity of OTA (Haschek *et al.*, 2002; Kumar *et al.*, 2007). OTA is thought to be the causative factor in endemic Balkan nephropathy of humans (Maxie and Newman, 2008). Ochratoxin toxicosis has been reported in a variety of animal species including swine, ducklings, horses, chickens, rabbits, turkeys, dogs and fish (Kitchen *et al.*, 1977; Haschek *et al.*, 2002; Kumar *et al.*, 2007; El-Sayed *et al.*, 2009). Cattle are thought to be resistant due to degradation of ochratoxins within the rumen, although pre-ruminant calves are susceptible (Haschek *et al.*, 2002). In domestic animals, ochratoxicosis is most commonly reported in pigs and chickens as a subchronic to chronic disease affecting growth and production (Maxie and Newman, 2008; Sakthivelan and Sudhakar Rao, 2010). Pigs are considered to be the most sensitive species, with chronic toxicosis occurring following ingestion of diets containing 0.2–4ppm OTA, while broiler chickens develop toxicosis at 2ppm OTA (Haschek *et al.*, 2002). The renal lesions in pigs consist of proximal tubular degeneration and atrophy with interstitial fibrosis and infiltration by mononuclear cells. Cystic dilatation of degenerated tubules occurs, which can lend a pitted, irregular appearance to the renal capsule, and glomerular hyalinization has been reported in severe cases. In acute cases, perirenal edema may occur (Maxie and Newman, 2008). Additional lesions of OTA toxicosis in pigs include multifocal necrotizing enteritis, fatty degeneration of the liver and lymphoid necrosis (Szczzech *et al.*, 1973). In broilers, OTA causes renal edema and renal tubular necrosis (Huff *et al.*, 1975). OTA is teratogenic,

mutagenic and carcinogenic, inducing renal tumors in rats (Haschek *et al.*, 2002).

### Citrinin

Citrinin is produced by toxic strains of several *Penicillium*, *Aspergillus* and *Monascus* spp. (Bennett and Klich, 2003), and has been associated with rice, wheat, oats, rye, corn, barley, vegetarian foods colored with red *Monascus* pigments and naturally fermented sausages from Italy. Citrinin is often found in association with OTA and is less toxic than OTA (Kitabatake *et al.*, 1993). Dogs receiving citrinin at dosages of 80 micromoles/kg intravenously or 10mg/kg orally developed proteinuria and glucosuria and ultrastructural changes in the epithelium of the S<sub>2</sub> segment, thick ascending limb, distal convoluted tubule and collecting ducts (Kitchen *et al.*, 1977; Krejci *et al.*, 1996). Dogs given oral 10mg/kg citrinin twice at 24 hour intervals developed similar lesions and developed glucosuria, proteinuria and granular casts in the urine; glucosuria was the earliest abnormality noted and persisted for 5 days, as did proteinuria (Kogika *et al.*, 1993). Cylindruria persisted from days 1 to 15, and renal values (BUN, creatinine) were elevated from days 2 to 5. Clinical signs in dogs administered 10mg/kg citrinin per day for 14 days included anorexia, retching, tenesmus, weight loss, prostration and death. Citrinin is teratogenic, embryotoxic and a putative carcinogen.

## Drugs

### Aminoglycoside antibiotics

Aminoglycoside antibiotics exert their nephrotoxic effects through induction of apoptosis and necrosis within the renal tubular epithelial cells of the proximal convoluted tubule as well as the distal tubules and collecting ducts (Lopez-Novoa *et al.*, 2011). Within these cells, aminoglycosides internalized through endocytosis accumulate in lysosomes, Golgi apparatus and endoplasmic reticulum. As the aminoglycoside concentration increases beyond a threshold level, the endosomal membranes become disrupted and the drug leaks into the cytosol where it activates the intrinsic apoptotic pathway, increases production of reactive oxygen intermediates, and interacts with mitochondria to interrupt the respiratory chain. Interaction with endoplasmic reticulum and Golgi apparatus result in dysfunction in protein synthesis and packaging. In sub-lethally affected cells, aminoglycosides interfere with cell membrane transporter systems, altering tubular reabsorption and threatening epithelial cell viability. Cellular necrosis and apoptosis result in the accumulation of cellular residues in the tubular lumen, causing obstruction which increases the intratubular hydrostatic pressure. This

back-pressure results in leakage of ultrafiltrate into the interstitium and peritubular capillaries and reduces the glomerular filtration pressure gradient, resulting in decrease in GFR. Aminoglycosides also have some direct glomerular effects including mesangial contraction, mesangial cell proliferation and alteration of filtration barrier selectivity. Toxicosis from aminoglycosides results in loss of urine concentration ability, polyuria, proteinuria, hematuria, cylindruria, azotemia and acute renal failure. In spite of the significant renal injury that may develop due to aminoglycoside exposure, the renal effects are generally considered reversible once the drug is withdrawn (Maxie and Newman, 2008). Additional adverse effects of aminoglycoside antibiotics include ototoxicity and induction of neuromuscular blockade.

### NSAIDs

Non-steroidal anti-inflammatory drugs are the most widely used analgesics in veterinary medicine and are the most common drugs involved in accidental overdoses in companion animals (Sebastian *et al.*, 2007; Gwaltney-Brant, 2007). In both therapeutic and overdose situations, the potential of most NSAIDs to have deleterious effects on the kidney must be considered. The adverse effects of NSAIDs on the kidney result from the inhibition of cyclooxygenases (COX) which results in decreased synthesis of prostaglandins (PGs). COX has two distinct isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues through the body while COX-2 is normally expressed in tissues at low levels, but can be induced in the presence of pro-inflammatory mediators that are expressed in sites of inflammation, pain or injury (Radi, 2009). COX-1 is the most abundant isoform expressed in the kidneys, and is located in the renal vasculature, collecting ducts and papillary interstitial cells. COX-2 is minimally expressed in the kidney and its localization within the different areas and impact on production of PGs in the kidney is species and maturation dependent. The kidney is a major site of PG synthesis, and PGs exert a variety of diverse functions within the kidney, including modulation of renal blood flow and GFR, regulation of sodium excretion and influencing renin release. PGs produced through the action of COX-1 are considered "protective," as inhibition of these PGs is associated with the majority of adverse effects from NSAIDs. NSAIDs such as ibuprofen are non-selective inhibitors of both COX isoforms, while newer NSAIDs may target COX-2, sparing COX-1 and resulting in fewer gastrointestinal and renal adverse effects (Khan and Alden, 2002). However, COX selectivity can be lost in overdose situations. The primary impact of COX-induced inhibition of PG synthesis in the kidney is reduction of renal blood flow, resulting in ischemia. The medulla and renal papillae



are at increased risk for NSAID-induced hypoxic injury due to their low oxygenation and relatively slow blood flow that predisposes to accumulation of toxic substances (Radi, 2009). Dogs, rats, mice and pigs are thought to be most sensitive to NSAID-induced papillary necrosis (Khan and Alden, 2002). Clinical effects of NSAID-induced renal injury include polyuria, polydipsia, dehydration, electrolyte imbalances and azotemia. Acute renal failure, interstitial nephritis and nephrotic syndrome have also been reported. Non-renal effects of NSAIDs include gastrointestinal ulceration.

## Metals

### Cadmium

The primary sources of exposure to cadmium are due to contamination of food, water and air. Cadmium in soil can be taken up by plants, and cadmium in water can bioaccumulate in shellfish. Inhalation exposure to cadmium can occur through exposure to industrial exhaust, fossil fuel combustion products and cigarette smoke; the latter is one of the major non-occupational sources of inhaled cadmium in humans (Gwaltney-Brant, 2002). Acute cadmium toxicosis primarily manifests as pulmonary injury, while nephrotoxicity resulting from cadmium is most commonly due to chronic exposure. Cadmium is poorly absorbed via the gastrointestinal tract, with less than 5% of ingested cadmium being absorbed; however, inhaled cadmium is more readily absorbed into the blood, where it binds to metallothionein, a 6800 dalton, cysteine-rich protein. Metallothionein transports cadmium primarily to the kidney and liver, with lesser amounts accumulating in bone and testicle. The cadmium-metallothionein complex is filtered through the glomerulus and reabsorbed from the filtrate into the proximal renal tubules by endocytosis (Khan and Alden, 2002). Within the phagolysosome, metallothionein is hydrolyzed, which releases the cadmium; the free cadmium triggers the *de novo* synthesis of additional metallothionein. Once the level of intracellular cadmium exceeds a species-specific threshold of tolerance (10–200 mcg/g wet weight), cellular injury progresses. Cadmium-injured cells have decreased reabsorptive capacity and decreased ability to concentrate urine (Gwaltney-Brant, 2002). Low molecular weight proteinuria (particularly  $\beta_2$ -microglobulinuria), amino aciduria, calciuria and glucosuria develop. Renal lesions include proximal tubular cell degeneration and necrosis, granular casts, hyaline casts, tubular atrophy, interstitial inflammation and interstitial fibrosis. Additional chronic renal lesions include fatty degeneration of pars recta tubular epithelium, nephrocalcinosis and glomerular disease resembling immune

complex glomerulonephritis. Extra-renal effects of cadmium include osteoporosis and osteopenia, hepatocellular necrosis, myocardial injury and testicular injury. Cadmium is a mutagen and putative carcinogen.

### Lead

The incidence of lead toxicosis has declined in the United States since the banning of lead-based paints in residential domiciles in 1977 and leaded gasoline in 1996 (with significant reductions in use of leaded gasoline for the prior 20 years). However, lead is still present in the paint of many older homes as well as common household items such as toys, artists' paints, linoleum, lead weights, lead fishing sinkers and ornaments. Additionally, despite bans on the use of lead shot for hunting waterfowl lead ammunition is still widely used on upland game, and lead toxicosis is still a significant cause of death among scavengers such as eagles and condors (Hunt *et al.*, 2009; Stauber *et al.*, 2010). Birds with lead toxicosis frequently present with emaciation and evidence of renal tubular degeneration (Pattee *et al.*, 2006). Acute lead toxicosis is most commonly associated with neurological signs in mammals, while chronic lead toxicosis can result in dysfunction of a variety of organ systems including gastrointestinal, neuromuscular, central nervous, hematological and renal (Gwaltney-Brant, 2002). Degeneration and necrosis of proximal renal tubules results in oliguria, amino aciduria, glucosuria and altered tubular ion transport. Acute renal failure may occur following exposure to lead, but chronic renal failure is more common. Dense, homogeneous, eosinophilic intranuclear inclusion bodies may be visualized in affected renal tubular cells; although suggestive for lead toxicosis, these inclusions are not pathognomonic as they can occur following exposure to other metals such as bismuth.

## Plants

### *Amaranthus* spp.

The pigweed family includes several different species capable of producing toxicosis, but *Amaranthus retroflexus* is the species most commonly associated with disease in domestic animals (Burrows and Tyrl, 2001). Renal injury from pigweed has been reported in pigs, cattle and sheep. Other herbivorous species are likely susceptible, although the disease was not able to be reproduced in rabbits fed *A. retroflexus* (Schamber and Misek, 1985). The toxic principle is not known. Although pigweeds do contain some oxalates, the levels are low and the clinical syndrome and lesions caused by pigweed toxicosis are not consistent with those seen in oxalate nephrosis (Burrows and Tyrl, 2001). Clinical signs generally begin

following several days of ingesting the plant, although renal lesions have been identified within 24 hours of ingestion. Clinical signs include weakness, ataxia, knuckling of pasterns, recumbency, paralysis and coma; death occurs within 1–2 days of onset of signs. Gross lesions of pigweed toxicosis include widespread edema, most prominently around the kidneys, rectum and omentum. Kidneys are pale and normal to swollen in size. Histopathologic changes within the kidney include interstitial edema, scattered hemorrhages and proximal tubular degeneration and necrosis. Dilated tubules and tubules filled with necrotic debris are often present and interstitial fibrosis may be present in chronic cases. Extra-renal lesions include edema and ulceration of the digestive tract.

#### *Lilium spp. and Hemerocallis spp.*

Ingestion of some species of the genera *Lilium* and *Hemerocallis* (Table 18.1) by cats has resulted in a potentially fatal renal dysfunction that has not been successfully reproduced in other species including dogs, rats and rabbits (Rumbeiha *et al.*, 2004). Although the toxic principle remains unknown, Rumbeiha *et al.* were able to demonstrate that the toxic effects originated from an aqueous floral extract of the Easter lily. All parts of the plant, including pollen, are toxic, and even small ingestions can result in significant renal injury (Fitzgerald, 2010). Prevention of serious renal injury can often be achieved if aggressive intravenous fluid therapy is instituted within the first 18 hours following exposure. Clinical effects include vomiting, depression, polyuria, polydipsia, azotemia, glucosuria, proteinuria and isosthenuria. Serum creatinine levels tend to elevate disproportionately to blood urea nitrogen. Histopathologic lesions include proximal convoluted tubule degeneration and necrosis with denudation of basement membrane and filling of tubular lumens with cellular debris (Rumbeiha *et al.*, 2004). Extra-renal lesions include vacuolar degeneration of pancreatic acinar cells.

#### *Quercus spp.*

Oak foliage and acorns provide forage to a variety of wildlife and livestock, but when oak is the primary source of feed for more than a few days, toxicosis can develop (Burrows and Tyrl, 2001). Oak buds, acorns and young leaves are involved in the majority of reported livestock poisonings and most oak species have, at one time or another, been implicated in animal poisonings. Cattle are most commonly affected, and reports in other species such as horses, goats and sheep are rare. The primary toxic principles in oak are tannins, phenolic and polyphenolic compounds such as ellagic and gallic acids, and tannin metabolites such as pyrogallol. At toxic doses, oak tannins precipitate proteins in the digestive

tract, causing erosions and ulcerations as well as altering mucosal absorption barriers. Increased absorption of polyphenolic tannins via injured mucosa results in damage to the liver and kidney; in rats, gallic acid was identified as the compound responsible for renal tubular necrosis (Harris *et al.*, 1966). Clinical signs begin 2 days to a week or longer after animals have been consuming large amounts of oak. Affected cattle initially develop anorexia, depression, rumen stasis and constipation which are followed by diarrhea (+/– blood), dehydration, colic, polyuria and subcutaneous edema of ventral areas such as neck, brisket, abdomen and perineum (Panciera, 1978). Clinical pathologic evaluation of serum reveals metabolic acidosis, elevations in BUN and serum creatinine, and electrolyte abnormalities (e.g., hyperkalemia). Cattle with severe renal injury become weak and die, while less severely affected animals may survive, although many will become chronically debilitated due to persistent renal insufficiency (Burrows and Tyrl, 2001). The disease progression is similar in other species, though sheep and goats do not develop appreciable edema and horses tend to have more severe diarrhea (+/– blood), colic and tenesmus. Gross findings include fluid accumulations within body cavities, subcutaneous edema, mesenteric edema and retroperitoneal edema (especially perirenal). Kidneys are swollen, pale and may have petechiae within the cortex; chronic cases may have shrunken, irregular and fibrotic kidneys. Extra-renal lesions include edema, congestion, erosions and ulcerations of the alimentary tract mucosa and some cases may have hepatocellular degeneration.

#### *Vitis spp.*

Ingestion of members of *Vitis* spp., including grapes, raisins and Zante currants, has been associated with acute renal failure in dogs (Morrow *et al.*, 2005) and, anecdotally, cats and ferrets (ASPCA Animal Poison Control Center, unpublished data). The toxic principle is unknown, but attempts to identify known nephrotoxins such as mycotoxins, pesticides, vitamin D<sub>3</sub> and heavy metals have been unsuccessful. Affected dogs generally demonstrate vomiting and/or diarrhea within 12 hours of ingestion of grapes or raisins, accompanied by anorexia, lethargy and abdominal discomfort in some cases (Morrow *et al.*, 2005). Severely affected dogs rapidly progress to anuria, and death or euthanasia occurs within 23–289 hours. Histopathological changes in the kidney include renal tubular degeneration and necrosis, primarily in the proximal tubules. Extensive sloughing of proximal tubule epithelium results in extensive necrotic debris within tubular lumens. Extra-renal lesions consist of centrilobular hepatic degeneration or necrosis, myocardial necrosis, soft tissue mineralization and fibrinous arteritis in colon, myocardium and aorta.

## Miscellaneous

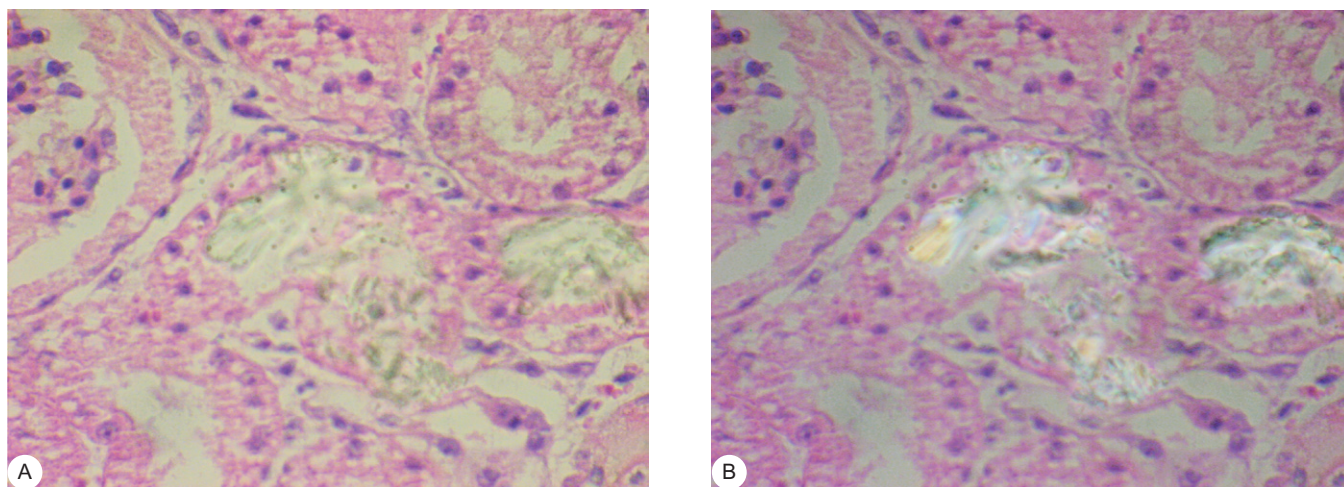
### Ethylene glycol

The most common cause of ethylene glycol toxicosis in domestic animals is via the consumption of automotive antifreeze, and dogs and cats are the most commonly affected species, although all mammals are susceptible to toxicosis. Ethylene glycol intoxication has three distinct stages: (1) initial CNS depression and derangement due to an alcohol-like effect on the brain, (2) profound metabolic acidosis with significant cardiorespiratory effects due to formation of acidic metabolites of ethylene glycol and (3) acute renal failure due to renal tubular injury (Thrall *et al.*, 2006). Renal tubular injury is due to direct action of the nephrotoxic metabolites glycoaldehyde and glyoxylate on the renal tubules as well as mechanical injury and obstruction by calcium oxalate crystals (Thrall *et al.*, 2006; Maxie and Newman, 2007). Calcium oxalate crystalluria is considered by some to be a hallmark of ethylene glycol, but crystalluria is present in less than half of all intoxications. Therefore, the absence of oxalate crystals does not eliminate ethylene glycol as a potential toxicant. Renal changes associated with ethylene glycol intoxication include pale, swollen kidneys, which may be gritty when cut. Microscopically, proximal tubular degeneration and necrosis is associated with the deposition of birefringent, light yellow crystals arranged in sheaves, rosettes or prisms (Maxie and Newman, 2007). Oxalate crystals may also be found within tubular lumens, within renal tubular epithelial cells and within the interstitium (Figure 18.2). Animals surviving more than a few days may show areas of tubular regeneration and/or interstitial fibrosis. Extra-renal lesions may

include deposition of oxalate crystals within small vessels of the brain, heart, liver and intestinal mucosa.

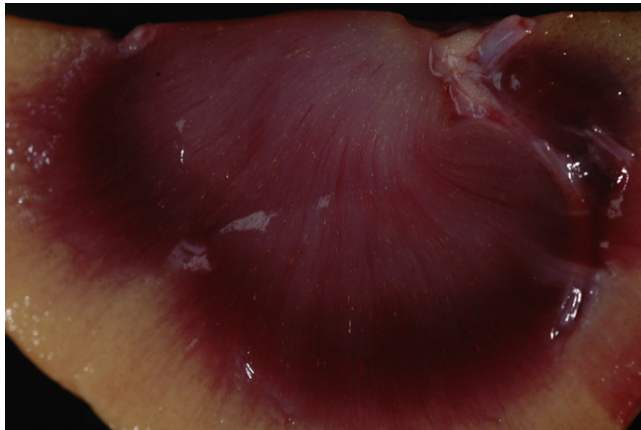
### Melamine:cyanuric acid

In North America in 2007, the discovery of renal injury in dogs and cats ingesting commercial pet foods led to the recall of over 60 million cans and pouches of “cuts and gravy”-style pet food contaminated with melamine and cyanuric acid. The contamination was found to have originated in wheat gluten from China, where the melamine had been added to artificially inflate the apparent protein level of the product. Cyanuric acid is a hydrolysis product of melamine that may also have either been intentionally added or coincidentally present as a melamine by-product (Puschner *et al.*, 2007). Subsequent to the pet food recall of 2007, the contamination of various food sources by melamine has been identified in several countries around the world including Italy, China, Spain, Korea, Taiwan and other Asian countries (Brown *et al.*, 2007; Gonzalez *et al.*, 2009; Yhee *et al.*, 2009; Brown and Brown, 2010; Cocch *et al.*, 2010). By themselves, melamine and cyanuric acid are of low toxicity, but in combination these compounds precipitate in acidic environments to form melamine cyanurate crystals (MCA) (Puschner *et al.*, 2007). In animals ingesting feed containing melamine and cyanuric acid, precipitation of MCA in the renal tubules has been associated with tubular injury, interstitial edema and renal failure. The exact mechanism of renal injury is not known, although mechanical injury from deposition of the large crystals is likely contributory. In cats, acute renal failure was induced within 48 hours following oral administration of 32 mg/kg



**FIGURE 18.2** Ethylene glycol poisoning, canine kidney. A. Tubules are lined by degenerate and attenuated epithelium and contain crystalline material consistent with calcium oxalate crystals. H&E stain, 100 ×. (Photo courtesy Thomas Brant, Mahomet, IL.) B. Tubules contain birefringent crystalline material consistent with calcium oxalate crystals. H&E stain, 100 ×, utilizing polarized light. (Courtesy Thomas Brant, Mahomet, IL.)



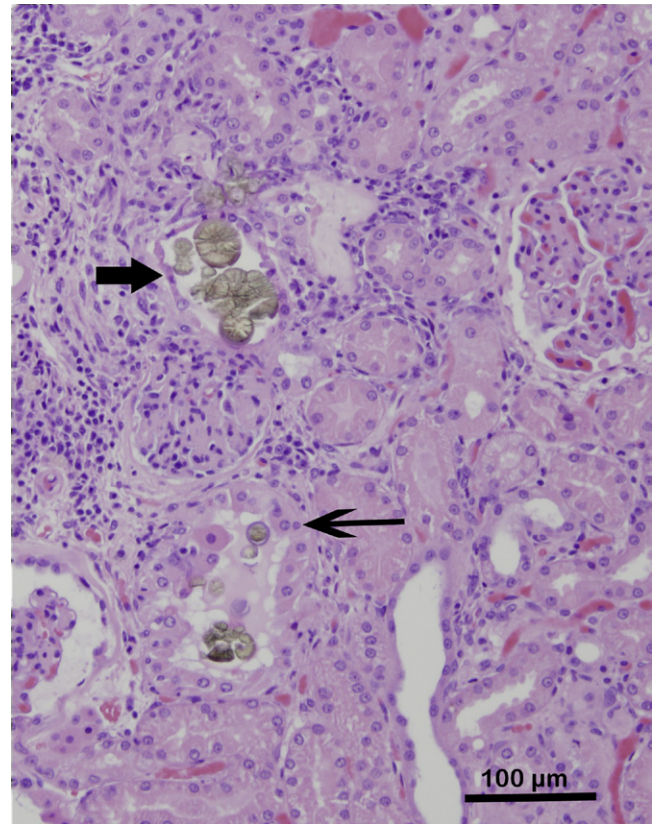


**FIGURE 18.3** Melamine:cyanurate crystals, feline kidney. The corticomedullary junction is obscured by a broad, dark red band of hemorrhage. Fine dots of 0.1 cm long threads of an opaque, variably white to yellow substance (i.e., crystals) are observed. These crystals extend from the papilla of the medulla into the obscured corticomedullary junction and are in parallel with the medullary rays. They are most concentrated at the renal crest. (Courtesy of Drs. Patricia Pesavento and Birgit Puschner, University of California, Davis.).

of melamine and cyanuric acid. MCA-induced nephropathy has been documented in humans, pigs, dogs, cats, fish, raccoon dogs and rats (Reimschuessel *et al.*, 2008; Bhalla *et al.*, 2009; Gonzalez *et al.*, 2009; Yhee *et al.*, 2009; Brown and Brown, 2010), although most species are likely susceptible. Affected animals develop clinical signs typical of renal injury: vomiting, anorexia, lethargy, polyuria/oliguria and dehydration. Renal lesions include hemorrhage and edema of the interstitium, predominantly in the medullary region (Figure 18.3). Degeneration and necrosis of the distal straight tubules is associated with the presence of large, translucent, pale yellow to brown, fan-shaped to starburst to globular crystals (Figure 18.4) (Puschner *et al.*, 2007). Crystals are also present in collecting ducts. The presence of these crystals in the more distal tubules, in addition to their morphology, can help to distinguish MCA from calcium oxalate crystals, which are found in the proximal tubules and tend to be smaller with variable shapes and sizes.

## CONCLUSIONS

The vital role that the kidney plays in maintaining homeostasis makes renal injury due to nephrotoxic agents a serious and potentially life-threatening problem. In many instances, prevention of renal injury can be achieved through proper gastrointestinal decontamination and implementation of aggressive intravenous



**FIGURE 18.4** Melamine:cyanurate crystals, canine kidney. Renal tubular epithelium is attenuated and the lumen is expanded by variably sized, fan-shaped to starburst, gray-brown crystals (thick arrow). Another tubule has attenuated epithelium and detached and necrotic cells, proteinaceous fluid and smaller crystals within the lumen (thin arrow). Inflammatory cells are present within the adjacent interstitium. H&E, Bar = 100 μm. (Courtesy of University of California, Davis Anatomic Pathology.).

fluid therapy following exposure to nephrotoxicants. Once renal injury has developed, veterinary intervention is essential in order to support the patient through the acute crisis and to allow time for regeneration and repair of injured renal tissues. Depending on the extent of injury and degree of regeneration, the patient may fully recover, may be left with residual renal insufficiency or may progress to chronic renal failure.

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# Reproductive toxicity and endocrine disruption

Tim J. Evans

## INTRODUCTION

Reproduction is a critical biological process in all living systems and is required for species survival. Toxicant-induced abortions, congenital defects and infertility can have devastating effects on livestock production. Wildlife species living in environments contaminated by industrial and/or agricultural chemicals have experienced impaired fertility and declining populations. There is growing concern within the scientific community and among government regulatory agencies about the effects of occupational and environmental exposures to reproductive toxicants on human fertility.

For the purposes of this chapter, the term “reproduction” will be used primarily in reference to vertebrate species of animals (especially mammals) and will be inclusive of “development,” which is sometimes treated as a separate topic in toxicology texts. This particular book chapter will emphasize the interactions between toxicants and the male and female reproductive tracts, as well as xenobiotic-induced effects on the growth, maturation and sexual differentiation of the embryo and fetus. Since “endocrine disruption” is an extremely common mechanism of action for xenobiotics associated with impaired reproductive function, reproductive toxicity and endocrine disruption will be discussed together in this chapter. Efforts will be made to clarify the current terminology related to reproductive toxicity and endocrine disruption and to introduce the reader to normal reproductive anatomy and physiology, as well as important concepts associated with embryonic and fetal development. Endocrine disruption

in wildlife species and humans and the effects of xenobiotics, including endocrine disrupting chemicals (EDCs), on reproductive function in domestic animals will be discussed along with a brief description of proposed mechanisms of action and the effects of some selected reproductive toxicants.

Unfortunately, space constraints limit the amount of information which can be presented in this chapter, and many of the presented topics cannot be discussed at great length. There are a number of recently published textbooks and issues of journals which cover some of these subjects in greater detail and provide information which is complementary to what is presented in this chapter (Burrows and Tyrl, 2001; Plumlee, 2004; Naz, 2005; Golub, 2006a; Hood, 2006; Jobling and Tyler, 2006; Jørgensen *et al.*, 2006b; Mukerjee, 2006; Gupta, 2011). The reader is directed to these publications and other references cited in this chapter in order to gain additional insight into specific areas of reproductive function and toxicology.

It is important that the reader understand that the areas of toxicology involving reproductive toxicity and endocrine disruption, in particular, are in continual flux. New data and exceptions to “classical” mechanisms of action are being reported on a regular basis, and there continues to be ongoing debate about the various aspects of normal as well as xenobiotic-induced abnormal reproductive function. Every effort has been made to accurately represent what is currently understood about the topics of discussion in this chapter. Controversial topics or those currently still subject to debate within the scientific community have been noted wherever possible.

## IMPORTANT DEFINITIONS AND CONCEPTS

### Reproduction

Reproduction in domestic, wild and laboratory vertebrates encompasses the wide range of physiological processes and associated behaviors and anatomical structures involved in the production of the next generation and the survival of a given species of animal (Senger, 2003). The physiological processes involved in reproduction generally include the following: (1) gametogenesis (production of sperm or ova) and the pre- and peri-pubertal changes leading up to its onset; (2) release of gametes (i.e., sperm transport and maturation, penile erection and ejaculation of sperm (mammals), copulation between a male and a female of the same species (several vertebrate classes) and ovulation of oocytes); (3) formation of the zygote (i.e., sperm storage, capacitation and other processes leading to fertilization, or union, of a single sperm with an egg); (4) embryonic and fetal development during the incubation process in egg-bearing vertebrates or, especially in the case of mammals, during pregnancy (gestation) (i.e., activities related to the initiation and progression of zygote cleavage, blastocyst formation, separation of the germ layers, placentation (mammalian species), neurulation and organogenesis (including sexual differentiation)); and finally (5) "birth" of a single or multiple offspring (hatching in oviparous vertebrates). In the conventional sense, the reproductive process culminates with birth or parturition (mammals); however, the initiation and maintenance of milk production (lactation) for the postpartum nutrition of offspring can also be considered a critical aspect of mammalian reproduction (Evans *et al.*, 2007; Evans and Ganjam, 2011).

### Reproductive toxicity

For the purposes of this chapter, "reproductive toxicity" will refer to any manifestations of xenobiotic exposure reflecting adverse effects on the physiological processes and associated behaviors and/or anatomical structures involved in animal reproduction or development. This is a fairly broad definition which encompasses developmental toxicity, as well as any toxic effects of post-pubertal exposures to xenobiotics on either male or female reproduction. "Developmental toxicity" refers to any adverse effect on the developing organism associated with either pre-conception parental exposures to toxicants or post-conception xenobiotic exposures to the embryo, fetus or pre-pubertal offspring (Hodgson *et al.*, 2000; Eaton and Klaassen, 2001; Foster and Gray, 2008). Adverse effects associated with developmental toxicity

of xenobiotics might not necessarily be observed until after the affected individuals have reached sexual maturity (Foster and Gray, 2008; Rogers and Kavlock, 2008).

### Teratogenesis

The term "teratogenesis" is derived from the Greek word for monster ("teras") and is a form of developmental toxicity (Panter, 2002; Rogers and Kavlock, 2008). "Teratogenesis" refers specifically to developmental defects induced by toxicant exposures occurring between conception and birth (Hodgson *et al.*, 2000; Eaton and Klaassen, 2001; Rogers and Kavlock, 2008). The types of abnormalities that are typically associated with teratogenesis include embryonic or fetal death; morphological, functional and/or neurobehavioral abnormalities; and decreased growth rate and/or birth weight (Panter, 2002; Rogers and Kavlock, 2008).

#### *Wilson's general principles of teratology*

With respect to teratogenesis, there are six basic tenets of teratology, first defined by Wilson in 1959, which need to be kept in mind whenever gestational exposure to a teratogenic xenobiotic is suspected or when a chemical is being evaluated for its teratogenic potential. As stated by Wilson in the *Handbook of Teratology* (Wilson, 1977), the general principles of teratology are as follows:

- 1 Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
- 2 Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
- 3 Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis.
- 4 The final manifestations of abnormal development are death, malformation, growth retardation and functional disorder.
- 5 The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).
- 6 Manifestations of deviant development increase in degree as dosage increases from no effect to the totally lethal level.

#### *Mechanisms of reproductive toxicity and teratogenesis*

There are a wide range of specific mechanisms of action by which xenobiotics can adversely affect reproductive function, including embryonic and fetal development. In general, many of these mechanisms are the same as those for toxicants affecting other organ systems and essentially involve some sort of toxicant-induced cellular dysregulation and alterations in cellular maintenance



which, when possible, the body attempts to repair, either successfully or unsuccessfully (Gregus, 2008). Oxidative damage and interference with normal enzymatic reactions are two common mechanisms by which xenobiotics can cause the dysregulation and altered maintenance of cells within various organs and tissues.

Normal reproduction and development require, by their very nature (see review in this chapter), signaling within and between a variety of diverse organs. In sexual reproduction and mammalian pregnancy, critical communication even takes place between distinctly different organisms (i.e., male and female and mother and offspring, respectively). The dependency of reproductive function on signaling pathways inclusive of gene transcription makes this physiological process especially prone to adverse effects associated with xenobiotic-induced disruption of or interference with cell-to-cell, organ-to-organ and/or even animal-to-animal communication. Many of the mechanisms which interfere with physiological signaling activity can be classified as forms of “endocrine disruption,” which will be discussed in much greater detail in this chapter.

There is a great deal of overlap between the various different mechanisms for reproductive toxicity. The level of exposure to a particular toxicant is an important determinant of what toxic effects are observed. Xenobiotics which “disrupt” endocrine pathways can do so without interactions with endogenous receptors, using mechanisms of action which can cause other forms of toxic insult at various dosages.

### *Reproductive toxicants and teratogens*

Any xenobiotic associated with adverse effects on development or male or female reproductive function can be classified as a “reproductive toxicant.” Xenobiotics capable of inducing teratogenesis are referred to as “teratogens.” Although any chemicals adversely affecting animal well-being have the potential to have a negative impact on development and reproductive function, this chapter will attempt to focus on mechanisms of actions and toxicants which specifically target normal embryonic or fetal growth and maturation or have a direct effect upon the male and/or female reproductive tract.

### **Hormones and hormone receptors**

The term “hormone” classically refers to a substance which is secreted into the circulation by a ductless gland and which alters the function of its target cells (Hodgson *et al.*, 2000). While the traditional “endocrine” aspect of hormone action involves organ-to-organ signaling (and in the case of mammalian pregnancy animal-to-animal signaling), it is recognized that hormones can also be

involved in “paracrine” (cell-to-cell) communication and signaling pathways within the same cell in which they were produced (“autocrine” function) (Evans *et al.*, 2007; Evans and Ganjam, 2011). In vertebrates there are a wide variety of different hormones involved in reproductive function. The major reproductive hormones are generally grouped according to their basic molecular structure and include amino acid derivatives (e.g., dopamine or prolactin inhibitory factor (PIF) and melatonin); peptides (e.g., oxytocin, adrenocorticotropin hormone (ACTH), corticotropin releasing factor or hormone (CRF or CRH), gonadotropin releasing hormone (GnRH) and thyrotropin releasing hormone (TRH)); proteins (e.g., activin, inhibin, insulin-like growth factors, prolactin and relaxin); glycoproteins (e.g., follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH or thyrotropin)); steroids (e.g., androgens, estrogens and progestagens); and eicosanoids, which include prostaglandins (Evans *et al.*, 2007; Evans and Ganjam, 2011).

The actions of hormones on their targets are generally mediated through receptors which initiate or inhibit some sort of signal transduction pathway or are required for hormone-induced alterations in gene expression. Hormone–receptor interactions can be modulated by a number of factors including the amount of hormone present, the affinity of the hormone for the receptor, receptor density and occupancy and interaction with other hormones, receptors and hormone–receptor complexes, as well as a variety of endogenous co-activators and inhibitors (Genuth, 2004a; Bigsby *et al.*, 2005). It should be clear by the end of this chapter that various xenobiotics are also capable, under certain exposure conditions, of modulating the interactions between endogenous hormones and their receptors.

### *Gonadal steroid hormones and their “nuclear” receptors*

The primary gonadal steroids (i.e., androgens and estrogens (some references also include progesterone)) are also referred to as the “sex” steroids, and the imitation and/or inhibition of the actions of these hormones by xenobiotics is what was first referred to as “endocrine disruption” (Krimsky, 2000; McLachlan, 2001). The major androgens (testosterone and dihydrotestosterone (5 $\alpha$ -reductase conversion product of testosterone in the testes and selected non-gonadal tissues)), estrogens (estradiol and estrone) and progesterone and endogenous progestagens facilitate the development and regulation of reproductive function in animal species, in large part by interacting with (i.e., functioning as ligands for) receptors which are members of the steroid/thyroid (“nuclear”) receptor superfamily, the largest family of transcription factors in eukaryotic systems (Tsai and O'Malley, 1994; Genuth, 2004a). Receptors in

this superfamily are large oligomeric proteins (Genuth, 2004a), which generally consist of five domains (A/B, C, D, E and F) (Tsai and O'Malley, 1994). Although specific portions of the gonadal steroid nuclear receptor molecules can interact with a variety of co-activators as well as inhibitors, the most important domains of these receptors are generally considered to be those involved in transactivation (*N*-terminal A/B domain; also C-terminus in estrogen receptors (ERs)); DNA-binding and hormone-receptor complex dimerization (middle portion containing two helical zinc fingers; C domain); and hormone (ligand) binding (C-terminus; E domain) (Genuth, 2004a; Bigsby *et al.*, 2005). While androgen, estrogen and progesterone receptors, which are members of the steroid/thyroid superfamily, are often thought of as being exclusively nuclear in their location, these receptors can also be located in the cytoplasm of some cells (Tsai and O'Malley, 1994; Genuth, 2004a). Cytoplasmic and nuclear gonadal steroid receptors can be bound to a variety of different heat shock proteins, which interact with the receptor's hormone-binding domain. Heat shock proteins can act as "blocking" molecules and are displaced by hormones binding to the receptors (Genuth, 2004a; Bigsby *et al.*, 2005) or as "chaperones" involved in receptor turnover and "trafficking" of these receptors between the nucleus and the cytoplasm (Pratt and Toft, 1997).

There is reportedly a single type of androgen receptor which is a member of the steroid/thyroid superfamily. In contrast, there are two types of nuclear ERs (ER $\alpha$  and ER $\beta$ ), which are the products of distinct genes on separate chromosomes (O'Donnell *et al.*, 2001). ER $\alpha$  and ER $\beta$  differ in their amino acid structure, tissue distribution, affinity for selective ER modulators (SERMs) and their role in female (Britt and Findlay, 2002) as well as, somewhat surprisingly, male fertility (O'Donnell *et al.*, 2001; Hess, 2003). The nuclear progesterone receptor also has two isoforms, progesterone receptor A and progesterone receptor B (PRA and PRB, respectively), which differ slightly in their amino acid sequences and their interactions with co-activators. However, unlike ER $\alpha$  and ER $\beta$ , PRA and PRB are the products of a single gene (Brayman *et al.*, 2006).

#### *Genomic and non-genomic mechanisms of action of gonadal steroid hormones*

Traditionally, the receptor-mediated reproductive effects of gonadal steroids were thought to occur almost exclusively through interactions between homodimers of the hormone-nuclear receptor complexes and specific regions of DNA upstream from the basal promoter of a given gene, referred to as hormone-response elements (HREs) or, more specifically, androgen and estrogen-response elements (ARE and ERE, respectively) (Tsai and

O'Malley, 1994; Genuth, 2004a). It is now understood that these "genomic" effects of gonadal steroids and their nuclear receptors, which involve alterations in gene transcription, can, in some instances, involve heterodimers of different nuclear steroid-receptor complexes, indirect binding of hormone-receptor complexes to DNA via proteins within a preformed transcriptional complexes and even ligand (hormone)-independent "activation" of nuclear gonadal steroid receptor molecules (O'Donnell *et al.*, 2001; Bigsby *et al.*, 2005; Thomas and Khan, 2005). In addition, it is also apparent that gonadal steroids can affect cellular function by non-genomic mechanisms of action involving changes in intracellular concentrations of ions, cAMP and its second messengers, and the mitogen-activated protein (MAP) kinase pathway. These non-genomic mechanisms are independent of the somewhat "time-consuming" alterations in gene expression traditionally associated with gonadal steroids and occur rapidly within seconds or minutes (O'Donnell *et al.*, 2001; Thomas and Khan, 2005). While the rapid, non-genomic effects of gonadal steroids most likely involve receptors bound to the plasma membrane, the specific identity and classification of these receptors remain unclear and might involve a number of different receptor types (Razandi *et al.*, 1999; O'Donnell *et al.*, 2001; Thomas and Khan, 2005; Warner and Gustafsson, 2006).

#### **Endocrine disruption**

"Endocrine disruption" is a developing, multidisciplinary area of research, involving aspects of both toxicology and endocrinology (McLachlan, 2001). "Endocrine disruption" is also a potential mechanism of action for many toxicants, and this term has been defined in a variety of different ways, depending on the circumstances and the intended audience. Some of these definitions can be fairly "broad," such as the one which will be used in this chapter (see below). However, "endocrine disruption" can also be defined fairly narrowly with respect to toxicant origin (synthetic versus naturally occurring); source or site of toxicant exposure (environmental contamination versus occupational exposure); xenobiotic mechanism of action (receptor agonism and/or antagonism (see definition below) versus other mechanisms independent of direct interactions between xenobiotics and receptors); and/or the timing of exposure (prenatal versus postnatal exposures) (Krimsky, 2000, 2001). It is critically important for one to carefully define the context in which "endocrine disruption" is being used in order to clearly and accurately discuss one's research findings or opinions with toxicologists, physiologists, wildlife biologists, medical professionals, regulatory personnel, the popular press and/or the general public (McLachlan, 2001).

Although the imitation and/or inhibition of the actions of androgens and, especially, estrogens by xenobiotics is what was first referred to as “endocrine disruption,” both the multidisciplinary area of study and mechanism of action generally referred to as “endocrine disruption” have evolved over the years to encompass a wide range of specific mechanisms of action which can ultimately result in adverse effects on invertebrate and/or vertebrate animals (McLachlan, 2001). As scientists continue to investigate the effects of xenobiotics on biological systems, the paradigm of endocrine disruption will continue to “shift,” and a willingness to “step out of the box” and discuss endocrine disruption in a broader context will be necessary in order to participate in scientific discussions, to design future experiments and/or to make informed, medical or policy decisions based on “good” science (McLachlan, 2001; Guillette, 2006). For the purposes of this chapter “endocrine disruption” will refer to the effects of any synthetic or naturally occurring xenobiotic which can affect the endocrine system of exposed individuals (i.e., the balance of normal hormonal functions) and, as a result of exposure, cause physiological alterations (Keith, 1997; Hodgson *et al.*, 2000). Within the broad scope of this definition, reproduction, including prenatal and pre-pubertal development, certainly would be expected to be one of the physiological functions most profoundly affected by chemicals capable of endocrine disruption. In fact, it could be argued that the majority of reproductive toxicants interfere with endocrine function in one way or another. However, adverse effects on other, “non-reproductive” endocrine systems can also be associated with exposures to xenobiotics, and these “non-reproductive” effects need to be taken into consideration as well when describing the endocrine disruption associated with exposure to a given chemical (Guillette, 2006).

### ***Mechanisms of endocrine disruption***

Endocrine disruption encompasses a wide range of mechanisms of action which can ultimately result in adverse effects on animal species. The mechanisms of action involved in endocrine disruption can include effects which are mediated directly by interactions between the xenobiotic and an endogenous hormone receptor (i.e., the xenobiotic functions as a ligand for an endogenous receptor and a receptor–ligand complex is formed), as well as those adverse effects which alter hormonal functions without direct interactions between the toxicant and an endogenous receptor (Keith, 1997). In addition, it should be noted that some xenobiotics are capable of causing endocrine disruption by functioning as an endogenous hormone receptor ligand, as well as by mechanisms of action which are independent of the formation of a xenobiotic (ligand)–receptor complex.

### ***“Classic” receptor-mediated endocrine disruption***

“Classic” endocrine disruption can involve imitation or mimicry of the interactions between cellular receptors and endogenous hormones (i.e., receptor agonism) and/or a blockade or inhibition of the formation of receptor–hormone complexes (i.e., receptor antagonism) (McLachlan, 2001). With respect to gonadal steroids, both genomic and non-genomic physiological responses can be affected by this mimicry or blockade of endogenous hormone receptor-mediated activity (Thomas and Khan, 2005). Xenobiotics which mimic the actions of endogenous androgens or estrogens (i.e., gonadal steroid receptor agonists) are referred to, respectively, as being either “xenoandrogens” or “xenoestrogens.” Conversely, reproductive toxicants which inhibit or block endogenous estrogens or androgens from interacting with their respective receptors (i.e., gonadal receptor antagonists) are generally classified as “antiandrogens” or “antiestrogens.” Progestagens (“progestogens” or “progestins” in some literature) is a generic term for endogenous or synthetic compounds which interact with progesterone receptors, and there is evidence of increasing environmental contamination with these types of EDCs.

Some xenobiotics can act as receptor agonists or antagonists, depending on the circumstances or tissues involved. “Selective ER modulators” or “SERMs” refer to a class of xenobiotics which, although originally classified as antiestrogens, can function as either ER agonists or antagonists, depending on the tissue in which estrogen-dependent responses are being discussed (Dutertre and Smith, 2000; Katzenellenbogen and Katzenellenbogen, 2000). SERMs are particularly relevant with respect to observed differences in their binding affinities to ER $\alpha$  or ER $\beta$  and their development as therapeutic agents for different types of estrogen-responsive neoplasia.

### ***Endocrine disruption independent of receptor-mediated interactions***

Endocrine disruption which is independent of interactions between xenobiotics and endogenous hormone receptors can occur in a variety of different ways. Xenobiotic exposure can result in alterations in the number of hormone receptor sites (up- or down-regulation) or can cause direct or indirect hormone modifications which alter hormonal function (Keith, 1997). Xenobiotics can change the rate of synthesis or destruction of endogenous hormones and can alter how hormones are stored, how they are released into and/or transported within the circulation or even how they are eventually cleared from the body (Keith, 1997; Sikka *et al.*, 2005). Any xenobiotic which is toxic to organs or tissues producing hormones (e.g., testis and ovary) has the potential to decrease hormone synthesis and thereby indirectly cause endocrine disruption (Devine and Hoyer, 2005). It should also be noted that some of these



mechanisms of endocrine disruption are not necessarily exclusive of one another. A given xenobiotic can potentially disrupt the normal balance of hormonal function by more than one mechanism which is independent of direct interactions between the toxicant and an endogenous hormone receptor.

#### ***“Androgenic” and “estrogenic” effects of xenobiotics***

The terms “androgenic” and “estrogenic” and their antonyms “antiandrogenic” and “antiestrogenic” have been used in a number of different contexts. Some authors have used these terms to refer specifically to the agonistic and antagonistic receptor interactions of xenobiotics (Hodgson *et al.*, 2000). Because the precise mechanism of endocrine disruption of a given toxicant might not always be known or might involve multiple mechanisms of action, these terms have also been used in a more general sense, especially in livestock and wild-life species, to refer to phenotypic changes which were similar to or the opposite of the effects which would be expected with exposure to endogenous androgens or estrogens (Guillette, 2006). This type of general usage can be helpful in some instances but can also be confusing, given that xenoandrogens and progestagens frequently have the opposite phenotypic effects as xenoestrogens. For instance, the effects of estrogenic xenobiotics can be described as antiandrogenic or anti-progestagenic in some instances, while the effects of xenoandrogens and progestagens can be referred to as being antiestrogenic in nature. Further confusion can be associated with exposures to mixtures of chemicals having different phenotypic effects, as is often the case in instances of environmental contamination, or with exposures to xenobiotics having mixed antiestrogenic and antiandrogenic effects (i.e., methoxychlor). When the terms “androgenic,” “estrogenic” or their antonyms are used within this chapter, an attempt will be made to clearly denote the intended specific or general meaning of the terms in the context in which they are used. The discretionary use of the terms “feminization” and “masculinization,” as well as “defeminization” and “demasculinization,” can also, in some instances, help to clarify and/or describe the phenotypic effects of a chemical suspected of endocrine disruption.

#### ***Endocrine disrupting chemicals, endocrine disruptors and hormonally active agents***

Any reproductive toxicant capable of endocrine disruption can be considered an “EDC” or an “endocrine disruptor.” Obviously, this includes a large number of xenobiotics which are used in commercially available industrial, agricultural and pharmaceutical products, as well as naturally occurring toxicants produced by plants and fungi. An effort will be made later in this chapter to

discuss some of the xenobiotics most often associated with endocrine disrupting mechanisms of action.

Another term frequently used with respect to endocrine disruption, especially regarding xenobiotics which interact with endogenous hormone receptors, is “hormonally active agent” or “HAA.” In most instances, “endocrine disrupting chemical,” “endocrine disruptor” or “hormonally active agent” can be used interchangeably to discuss the actions of a given xenobiotic. However, whereas “endocrine disrupting chemical” and “endocrine disruptor” generally have negative connotations and imply, by virtue of the inclusion of the term “disrupt,” something “dangerous” and the likelihood of adverse or toxic effects, the term “hormonally active agent” is more benign and only indicates that a given xenobiotic has the potential to affect a hormonal pathway in an animal (Krimsky, 2001). As pointed out by Krimsky (2001), a mechanism rather than a specific pathology is inferred by “hormonally active,” and “hormonally active agent” is the nomenclature preferred by the National Research Council (Knobil, 1999), especially when referring to xenobiotics which interact with endogenous hormone receptors.

The circumstances and intended audience will often dictate the terms used to describe xenobiotics associated with or suspected of being having endocrine activity. “Environmental hormone” and “environmental signal” have also been used, along with “HAA,” “EDC” and endocrine disruptor, to describe xenobiotics capable of interacting with endogenous hormone receptors (McLachlan, 2001; McLachlan *et al.*, 2006). However, the context in which these two terms have been routinely used generally implies environmental contaminants with documented adverse endocrine effects on animals or humans. In some instances, the term “HAA” might be more “politically correct” (Krimsky, 2001) than “EDC,” “endocrine disruptor,” “environmental hormone” or “environmental signal” when discussing chemicals with a suspected hormonal activity that has not been clearly associated with adverse effects on animals in a research and/or clinical setting.

#### ***Aryl hydrocarbon receptor-mediated endocrine disruption***

Endocrine disruption mediated by the aryl hydrocarbon receptor (AhR) is a relatively complex, species- and tissue-dependent phenomenon, involving several of the previously described mechanisms of EDC action and interactions with many important, environmentally persistent compounds. Some aspects of AhR-mediated endocrine disruption are reminiscent of the ligand-induced transcription associated with gonadal steroid receptor function. However, the unique nature of the endogenous AhR and its interactions with primarily xenobiotic agonists warrants further discussion.



### *Aryl hydrocarbon receptor agonists*

The major agonists for the AhR protein belong to the class of environmental contaminants referred to collectively as “halogenated” or “polyhalogenated aromatic hydrocarbons” (HAHs or PAHs, respectively) and includes many highly stable and lipophilic organochlorine industrial chemicals (e.g., polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzodifurans (PCDFs)), as well as their metabolites (Safe, 2005). In addition, other organic compounds, such as polycyclic aromatic hydrocarbons (PAHs) (e.g., 3-methylcholanthrene and benzo[a]pyrene) (BaP)) and flavones (e.g.,  $\beta$ -naphthoflavone), have also been shown to be AhR agonists (Parkinson and Ogilvie, 2008).

### *Mechanisms of aryl hydrocarbon receptor-mediated endocrine disruption*

Many of the mechanisms of action mediated by AhR–ligand interactions have been elucidated using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as a prototypical AhR agonist (Safe, 2005; Parkinson and Ogilvie, 2008). TCDD is considered by many to be the most toxic of all of the HAHs, and it is reported to have the highest AhR-binding affinity of any of the xenobiotics in that class of chemicals (Safe, 2005). The AhR, also referred to as the “dioxin receptor,” is located in the cytoplasm bound to heat shock proteins (Parkinson and Ogilvie, 2008). Following ligand (i.e., TCDD) binding and the subsequent disassociation of the heat shock proteins, the AhR is activated by phosphorylation (Parkinson and Ogilvie, 2008) and the activated ligand–AhR complex undergoes a rapid sequence of events involving interactions with the AhR nuclear translocator protein (Arnt) and relocation of the ligand–AhR–Arnt complex into the nucleus (Safe, 2005).

Within the nucleus, the liganded AhR/Arnt heterodimer can facilitate a variety of endocrine disrupting mechanisms. This activated heterodimer complex can interact with dioxin/xenobiotic-response elements (DREs/XREs), which function in much the same way as the previously discussed HREs, and with various co-activators to increase the expression of selected genes (Safe, 2005; Parkinson and Ogilvie, 2008). Depending on the animal species and the tissue, multiple-phase I drug-metabolizing enzymes (e.g., cytochrome P450 (CYP) enzymes (CYP1A1, CYP1A2 and CYP1B1)) and enzymes involved in phase II drug-biotransformation reactions (e.g., glutathione-*S*-transferase and glucuronosyl transferase), are induced by TCDD (Safe, 2005).

Although the antiandrogenic and antiestrogenic properties of TCDD have been associated with the ability of HAHs to induce enzymes involved in androgen and estrogen metabolism, TCDD can interact with

androgen-, estrogen- and progestagen-modulated pathways in a number of ways, including interference with neuroendocrine development (Petersen *et al.*, 2006). AhR-mediated effects of TCDD can interfere with the biosynthesis of testosterone by a mechanism which alters the regulation of the synthesis and release of LH (Sikka *et al.*, 2005). It has also been shown in cell cultures that TCDD can disrupt testosterone signal transduction pathways (Jana *et al.*, 1999). The liganded AhR/Arnt heterodimer appears to be able to interact with inhibitory DREs (iDREs) in selected tissues to suppress the expression of some genes induced by estrogens (Safe, 2005), as well as be able to actually block the ability of estrogen–ER complexes to bind to their HREs (Kharat and Saatcioglu, 1996; Thomas and Khan, 2005). It is likely that a variety of other means of crosstalk between TCDD- and estrogen-mediated signaling pathways exist, and, in fact, TCDD has actually been shown to have the potential for estrogenic activity through interactions between liganded AhR/Arnt heterodimers and unliganded ERs (both ER $\alpha$  and ER $\beta$ ) (Ohtake *et al.*, 2003; Bigsby *et al.*, 2005; Thomas and Khan, 2005). Ohtake *et al.* (2003) have reported that these novel interactions resulted in the recruitment of unliganded ERs and p300 co-activator to gene promoters which are responsive to estrogens. Based on the results of the various experiments performed with TCDD, it is important to remember that the effects observed following exposure to HAHs and EDCs, in general, can be dependent on animal species involved, as well as the type of tissue, organ or physiological response being evaluated.

### *Epigenetic mechanisms of action of endocrine disrupting chemicals*

In recent years there has been increasing interest in the association between prenatal exposures to some reproductive toxicants and the postnatal development of neoplasia (cancer) involving the reproductive tract, as well as the occurrence of transgenerational or vertically transmitted adverse reproductive effects (Crews and McLachlan, 2006). These two phenomena are not mutually exclusive of one another, and, in fact, there is increasing evidence of vertically transmitted neoplasia involving reproductive organs (McLachlan *et al.*, 2006). Both tumor formation and transgenerational reproductive abnormalities can occur because of “genetic” mutations or alterations in the genotype (i.e., DNA sequence) or as a result of “epigenetic” changes where there are heritable modifications in the properties of a cell which do not represent genetic changes (inherited phenotypic alteration without genotypic change) (Lewin, 1998; McLachlan, 2001; Crews and McLachlan, 2006).

Epigenetic changes are a normal part of development and most likely represent one means for heritable

environmental adaptation (Crews and McLachlan, 2006). One of the more common mechanisms of epigenetic modification in mammals is DNA methylation of CpG nucleotides in the promoter regions of genes, which results in methylated genes being “turned off” and unmethylated or demethylated genes being “turned on” (McLachlan, 2001; Anway and Skinner, 2006). Patterns of DNA methylation are generally established during development at the gastrulation stage (i.e., lineage-specific pattern in somatic cells) and after sex determination (i.e., germ line-specific lineage pattern in the gonad) (Anway and Skinner, 2006). DNA methylation can facilitate “genomic imprinting,” a form of epigenetic gene regulation resulting in the expression of the allele from only one parent (i.e., monoallelic expression) (McLachlan, 2001; Anway and Skinner, 2006). The ability of developmental exposures to xenobiotics to provide a basis for adult disease, such as neoplasia, might very likely involve epigenetic changes involving methylation or demethylation of the promoters for specific genes (Newbold *et al.*, 2006). Epigenetic modification by alterations in DNA methylation patterns in the germ line might be one mechanism for observed xenobiotic-induced transgenerational (vertically transmitted) effects associated with infertility and tumor susceptibility in rodents (Anway and Skinner, 2006; Newbold *et al.*, 2006).

#### *Disruption of “non-reproductive” endocrine systems*

Although it can be argued that almost all endocrine systems are “reproductive” to some extent, there are multiple systems with primary functions which are not directly related to reproduction, and several of these systems have also been identified as potential targets of EDCs. In addition, gonadal steroids and xenobiotics which mimic these endogenous hormones can have “non-reproductive” effects. The synthesis of triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) by the thyroid gland can be decreased by chemicals which inhibit the uptake of iodine (e.g., perchlorate and thiocyanate) and also by xenobiotics which inhibit thyroperoxidase (e.g., thiourea, propylthiourea (PTU), some sulfonamides, methimazole, carbimazole, aminotriazole and acetoacetamide) (Capen, 2008). Polybrominated diphenyl ethers (PBDEs) have been shown to have antithyroidal activity (Guillette, 2006), and thyroid hormone secretion can be inhibited by exposure to excessive amounts of iodine or lithium (Capen, 2008). Xenobiotics, such as the *o,p'*-DDD metabolite of dichlorodiphenyltrichloroethane (DDT), can interfere with glucocorticoid metabolism (Guillette, 2006), and there has been increasing interest in the relationship between gestational and neonatal exposures to xenoestrogens and the development of obesity (Cooke and Naz, 2005; Newbold *et al.*, 2005, 2006). Some EDCs (e.g., organotin compounds) have recently been described as “obesogens”

because of their ability to affect adipogenesis by several different mechanisms, including interactions involving the isoforms of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) which play key roles in glucocorticoid metabolism (Grün and Blumberg, 2006). Because of increased societal concerns about obesity, there is likely to be greater future interest in organotins and other EDCs with similar “obesogenic” activities.

## NORMAL ANIMAL REPRODUCTION

Reproduction is a complex and dynamic process involving precise coordination and integration of the functions of multiple organs within the body. The production of viable and functional gametes and their transport and union to form a zygote which develops into a healthy and fertile individual require that many stringent physiological and metabolic needs be met. A thorough understanding of the mechanisms involved in reproduction is absolutely essential in order to recognize which steps in the reproductive process are most susceptible to the adverse effects of potential toxicants. It is critical that one be able to understand the pathophysiological basis for reproductive abnormalities. In addition, it is necessary, from a clinical perspective, to identify what constitutes “normal” reproduction in order to recognize abnormal reproductive behaviors and morphological changes in both domestic and wild animals. Impaired reproductive function in domestic animals, which is associated with exposure to toxic amounts of xenobiotics, necessitates the use of diagnostic, prognostic and therapeutic procedures which require a thorough knowledge of normal reproductive anatomy and physiology.

Normal reproduction will be reviewed in this chapter to provide a basis for discussion concerning specific reproductive toxicants. Although the emphasis will be on mammalian reproduction, many of the principles will be applicable to other classes of vertebrates. If additional information is needed, textbooks are available which provide a comprehensive overview of animal reproduction (Hafez and Hafez, 2000; Senger, 2003), as well as general veterinary anatomy (Dyce *et al.*, 2002). Other references can be consulted for descriptions of various aspects of normal reproduction in species of domestic or laboratory animals, which might be of particular interest to the reader (Johnston *et al.*, 2002; Hedrich and Bullock, 2004; Suckow *et al.*, 2006; Youngquist and Threlfall, 2007).

### Neuroendocrine control of reproduction

In humans and animals alike, visual, olfactory, auditory and other sensory data are integrated within the brain

and are reflected in endocrine events. The neuroendocrine functions of the pineal gland, hypothalamus and pituitary gland play an important role in the integration and endocrine regulation of the body's physiological processes and are potential targets for many reproductive toxicants (i.e., dioxins). These structures within the brain and proper function of the hypothalamic-pituitary-gonadal axis facilitate development of the reproductive tract and endocrine regulation of spermatogenesis in the male and the estrous or menstrual cycle in the female. The onset of puberty and sexual behavior in males and females, the ability to achieve erection and ejaculation in males, and the normal progression of gestation, parturition and lactation in females are all affected by the secretions of the hypothalamus and pituitary gland, as well as interactions between these structures and the reproductive tract (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011).

The hormones involved in the neuroendocrine control of reproduction are produced in several regions of the brain. Melatonin is produced in the pineal gland. The major hormones of reproductive interest which are of hypothalamic origin are dopamine, CRF, GnRH and TRH. Oxytocin is released from the posterior pituitary (neurohypophysis), and ACTH, FSH, LH, prolactin and TSH are synthesized and released from the anterior pituitary (adenohypophysis) (Ginther, 1992; Evans *et al.*, 2007; Evans and Ganjam, 2011). The production and release of these hormones are regulated by various positive and negative feedback loops, which are potentially susceptible to the effects of hormonally active xenobiotics.

## Puberty

### *The onset of puberty*

Puberty in male and female offspring implies reproductive competence and corresponds to the onset of normal spermatogenesis in the male and reproductive cyclicity in the female. Puberty can be indicated in the female by the age at first estrus or ovulation or even the age at which pregnancy can be maintained safely (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). In the male, the age at the time of preputial separation and the acquisition of the ability to ejaculate or the age at the first appearance of spermatozoa in the ejaculate or urine, as well as the production of threshold concentrations of fertile sperm in the ejaculate, have all been used as indicators of puberty (Senger, 2003). Species, nutritional status, environmental and social factors, pheromones and photoperiod in short- or long-day breeders can all influence the age of onset of puberty in animal species (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011).

### *The endocrinology of puberty*

From an endocrine perspective, puberty is associated with the ability of the hypothalamus to release enough GnRH to induce gonadotropin production by the anterior pituitary gland (Senger, 2003). This endocrine milestone is brought about by the postnatal developmental changes which allow the hypothalamus to overcome the negative feedback of testicular androgens and estrogens in males and which facilitate the ovary's ability to produce sufficient estrogens to induce the preovulatory surge of GnRH in females (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). Many of the endocrine changes which come into play with the onset of puberty are also involved in the transition from anestrus to the ovulatory season in seasonally polyestrous female animals (Evans *et al.*, 2007; Evans and Ganjam, 2011).

### *The susceptibility of the pubertal process to reproductive toxicants*

While puberty is often described simply in terms of a single, initial reproductive event (e.g., first estrus, ovulation or ejaculation), the attainment of reproductive competency is actually a process which is susceptible to the effects of reproductive toxicants. Xenobiotics can interfere with important physiological and morphological transformations necessary for the normal stepwise progression toward reproductive competency. Pre-pubertal follicular development, as well as the onset of the preovulatory LH surge in the female and the transition in testicular estrogen synthesis from the Sertoli cell to the Leydig cell in the males of many species, in addition to the postnatal proliferation of Sertoli cells in some mammals, are all potentially susceptible to the adverse effects of xenobiotics. Pre- or peri-pubertal exposure to hormonally active xenobiotics, such as anabolic steroids and antiandrogens, can interfere with postnatal reproductive development and function and can impair an animal's ability to reach its maximum reproductive potential (Monosson *et al.*, 1999; Evans *et al.*, 2007; Evans, 2011a; Evans and Ganjam, 2011).

## Normal male reproductive anatomy and physiology

### *Reproductive anatomy of the male*

Anatomical structures associated with reproduction in the male usually include, especially in mammals, paired testes (male gonads) positioned outside the abdominal cavity in most species; an excurrent duct system (i.e., efferent ductules, paired epididymides, vas deferens and urethra); accessory sex glands (i.e., ampullae, seminal vesicles, prostate and bulbourethral glands); a



scrotum and its associated thermoregulatory functions to protect the testes from mechanical and thermal insult and some form of copulatory organ or penis with a mechanism for protrusion, erection, emission of glandular secretions and ejaculation of sperm. The primary functions of the testis (testicle) are spermatogenesis (production of male gametes (sperm or spermatozoa)) and steroidogenesis (production of androgens and estrogens). Unlike the female in which oögonia are no longer replicating and the full complement of potential oocytes are present at birth, spermatogonia are proliferating and differentiating into spermatozoa continuously, and the testis is organized in such a way as to maximize sperm production (Senger, 2003; Evans and Ganjam, 2011).

#### *Testicular structure*

The parenchyma of the testis is divided into the tubular and interstitial compartments (Senger, 2003; Evans and Ganjam, 2011) (Figure 19.1a). The structural and functional units of the testis are the seminiferous tubules within the tubular compartment and the Leydig (interstitial cells) within the interstitial compartment. Depending on the species, it is estimated that the seminiferous tubules comprise approximately 80% of the adult testis, with the interstitium comprising most of the remaining 20% (Genuth, 2004b). Seminiferous tubules form highly convoluted loops (tubulus contortus) which begin and end with straight portions (tubulus rectus) that connect to the rete tubules (Thomas and Thomas, 2001; Genuth, 2004b; Senger, 2003; Evans and Ganjam, 2011). In some species, the rete tubules coalesce in a fibrous region of the testis referred to as the mediastinum, which joins with septal projections of the tunica albuginea, part of the testicular capsule (Senger, 2003). The rete tubules join with the efferent ductules which attach to the epididymidis.

Within the seminiferous tubules are germ cells at various stages of differentiation and Sertoli cells which provide germ cells with structural support and nutrients, as well as regulatory and paracrine factors (Thomas and Thomas, 2001) (Figure 19.1b). Tight junctions (junctional complexes) between adjacent Sertoli cells divide the seminiferous epithelium into basal and adluminal compartments, with Sertoli cells anchored to the basement membrane and surrounding the developing populations of germ cells (Thomas and Thomas, 2001; Genuth, 2004b; Senger, 2003; Evans and Ganjam, 2011). The seminiferous tubules are surrounded by peritubular myoid cells, which in combination with the junctional complexes, form the “blood–testis barrier” to prevent free exchange of large proteins and some xenobiotics between the blood and the fluid within the seminiferous tubules (Thomas and Thomas, 2001; Senger, 2003; Evans and Ganjam, 2011).

Within the interstitial compartment are the Leydig (interstitial) cells, as well as capillaries, lymphatic vessels and connective tissue (Senger, 2003; Evans and Ganjam, 2011). The Leydig cells are homologous to the theca interna cells in the ovary and produce testosterone (also estrogen in some species). There are species differences with respect to the abundance of Leydig cells in the interstitium, and these differences are important to recognize when reporting Leydig or interstitial cell hyperplasia in response to toxicant exposure. It should also be noted that Leydig and, to a lesser extent, Sertoli cells contain enzymes involved in xenobiotic biotransformation, and the synthesis of toxic metabolites can actually occur within the testis, in close proximity to the target cells for a given reproductive toxicant (Thomas and Thomas, 2001; Haschek *et al.*, 2010).

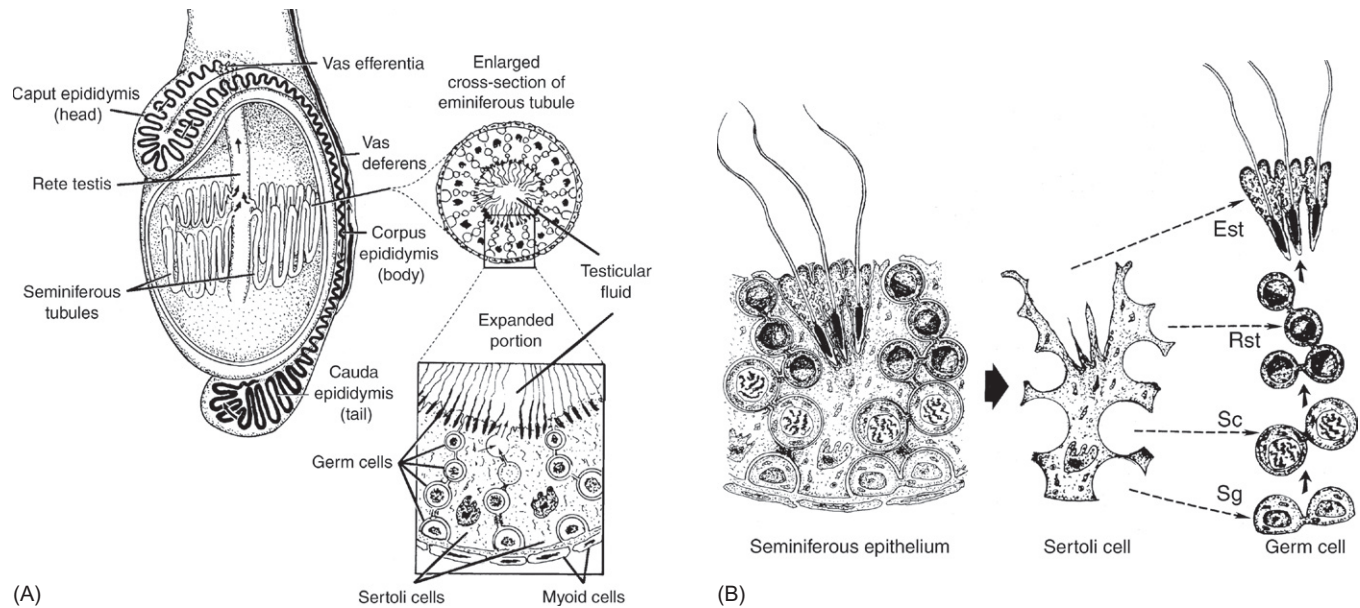
#### *Excurrent duct system*

The excurrent duct system consists of the efferent ductules, the epididymal duct and the ductus deferens. This duct system functions to conduct spermatozoa, rete fluid and some testicular secretory products away from the testis and eventually into the pelvic urethra (Senger, 2003; Evans and Ganjam, 2011). The reabsorption of fluid by a species-variable number of efferent ductules is essential for normal testicular function (O'Donnell *et al.*, 2001; Hess, 2003), and these tubules terminate by joining a single highly coiled epididymal duct, commonly referred to as the epididymidis or epididymis. Depending on the species, the epididymidis is generally subdivided into the initial segment, head (caput), body (corpus) and tail (cauda), with the various portions sometimes being further subdivided (França *et al.*, 2005). The primary functions of the epididymidis are transport and sustenance of sperm, reabsorption and secretion of fluid (initial segment and head, respectively); spermatozoal acquisition of motility and fertile potential (i.e., sperm maturation); recognition and elimination of defective spermatozoa; sperm storage prior to ejaculation and secretory contributions to the seminal fluid (Sutovsky *et al.*, 2001). The epididymal transit time varies somewhat with species, but is generally approximately 7 to 14 days in length, depending on several factors including ejaculation frequency. The ductus deferens conducts spermatozoa matured in the epididymidis to the pelvic urethra which helps to form the penis.

#### *Accessory sex glands*

There are a number of accessory sex glands (the complement of which varies with species) that contribute to the composition of the seminal fluid in mammals. These glands include the ampullae, seminal vesicles (vesicular glands), prostate and bulbourethral glands (Senger, 2003; Evans and Ganjam, 2011). Laboratory rodents (i.e., mice





**FIGURE 19.1** Loops of the seminiferous tubules, rete testis and excurrent duct system (i.e., efferent ductules (vas efferentia), epididymis (epididymis) and ductus deferens (vas deferens)), as well as a cross-section of a seminiferous tubule showing the microanatomy of the seminiferous epithelium in a “typical” mammalian testis, are shown in (A). Mature spermatozoa follow the pathway denoted by arrows. Testicular fluid is secreted by the Sertoli cell into the lumen of the seminiferous tubule. The portion of the testicular parenchyma outside of the seminiferous tubules is the interstitium. The predominant cell type within the interstitium is the Leydig or interstitial cell. The complex nature of the association between Sertoli cells and developing germ cells within the seminiferous epithelium in a “typical” mammalian testis is shown in (B). The Sertoli cell and germ cells are shown schematically disassociated to demonstrate how spermatozoal precursors occupy spaces between adjacent Sertoli cells. Spermatogonia, spermatocytes, round spermatids and elongate spermatids are denoted by Sg, Sc, RSt and Est, respectively. This figure was adapted, with permission, from [Garner DL, Hafez ESE \(2000\)](#) (modifications and artwork courtesy of Don Connor and Howard Wilson).

and rats) have an additional gland referred to as the preputial gland, which appears to have a role in the production of pheromone ([Haschek et al., 2010](#)). These accessory sex glands in the male are generally considered to be androgen dependent, with conversion of testosterone to DHT occurring in the prostate and seminal vesicles of many species ([Senger, 2003; Haschek et al., 2010; Evans and Ganjam, 2011](#)). The weights of the accessory sex glands can be used as an indirect measure of testosterone concentrations or exposure to antiandrogens ([Thomas and Thomas, 2001; Senger, 2003; Evans and Ganjam, 2011](#)).

#### External genitalia

The external genitalia of the male consist of the copulatory organ or penis, the prepuce, which protects the penis from environmental and mechanical injury, and the scrotum for testes positioned outside of the abdominal cavity. Penile structure is extremely species variable, with some species even having a special penile bone (i.e., os penis), but the shaft of the penis generally consists of erectile tissue (corpus cavernosum and corpus spongiosum) which surrounds the pelvic urethra. The glans penis is homologous to the female clitoris, and stimulation of the glans is the primary factor involved

in the initiation of ejaculation ([Senger, 2003; Evans and Ganjam, 2011](#)). The scrotum protects the testes from mechanical injury and, in conjunction with the tunica dartos, cremaster muscle and pampiniform plexus, plays a major thermoregulatory role with respect to temperature-sensitive, testicular spermatogenesis. In some species of wildlife (e.g., elephants and marine mammals), the testes are positioned intra-abdominally. Xenobiotics, which cause hyperthermia (i.e., ergopeptine alkaloids) or which induce fever, have the potential to adversely affect spermatogenesis.

#### Spermatogenesis

Spermatozoa are highly specialized haploid cells equipped with a self-powered flagellum to facilitate motility, as well as an acrosome to mediate penetration of the zona pellucida. Spermatogenesis takes place within the seminiferous tubules and consists of all the changes germ cells undergo in the seminiferous epithelium in order to produce adequate numbers of viable spermatozoa each day and to continuously replace spermatogonial stem cells ([Thomas and Thomas, 2001; Senger, 2003; Evans and Ganjam, 2011](#)). Spermatogenesis provides for genetic diversity and ensures that germ cells are in an immunologically favored site ([Senger,](#)

2003; Evans and Ganjam, 2011). The duration of spermatogenesis varies with species but generally ranges between 4 and 8 weeks (approximately 30–60 days) in domestic and laboratory animals and is approximately 75 days (almost 11 weeks) in humans. It is important to keep in mind the durations of spermatogenesis and epididymal sperm transport in a given species, as well as the normal, species-specific number of spermatozoa produced daily by the testes, when determining the period of toxicant exposure relative to the appearance of abnormal spermatozoa in an ejaculate and when assessing the severity and reversibility of toxicant-induced damage to sperm precursors within the testes.

Spermatogenesis can be subdivided into three phases or stages referred to as “proliferation,” “meiosis” and “differentiation.” During each of these phases, sperm precursors or male germ cells (spermatogonia, spermatocytes or spermatids) undergo specific, stepwise changes as they develop into spermatozoa which will eventually be released into the excurrent duct system. Each of these phases involves a different type of germ cell undergoing a different developmental process, and, as such, these phases have the potential to differ in their susceptibility to the mechanisms of action of various reproductive toxicants.

#### *Proliferation (mitosis or spermatocytogenesis)*

The “proliferation” phase of spermatogenesis has also been referred to as “mitosis” or “spermatocytogenesis” and occurs within the basal compartment of the seminiferous tubule. Proliferation denotes all of the mitotic divisions involving spermatogonia (Senger, 2003; Evans and Ganjam, 2011). A large number of B-spermatogonia result from the mitoses of several generations of spermatogonia (e.g., A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub>, A<sub>4</sub> and I; some species variations in nomenclature) (Senger, 2003; Genuth, 2004b; Evans and Ganjam, 2011). Stem cell renewal is accomplished during proliferation by the reversion of some spermatogonia to more primitive germ cells (Senger, 2003; Evans and Ganjam, 2011). Germ cell mitosis during spermatogenesis ends with the transformation of B-spermatogonia into primary spermatocytes, and this process is particularly susceptible to toxicants, such as chemotherapeutic agents and radiation, which target rapidly dividing cells.

#### *Meiosis*

“Meiosis” takes place within the adluminal compartment of the seminiferous tubules and involves the participation of primary and secondary spermatocytes in a total of two meiotic divisions. The chromosomal reduction, synapsis and crossover, as well as cellular division and separation, which occur during this phase of spermatogenesis, are extremely complex and guarantee

genetic diversity (Senger, 2003; Genuth, 2004b; Evans and Ganjam, 2011). The meiosis phase of spermatogenesis is considered by some to be most susceptible to toxic insult (Thomas and Thomas, 2001) and ends with the production of haploid round spermatids (Senger, 2003; Evans and Ganjam, 2011).

#### *Differentiation (spermiogenesis)*

Spermatozoa have been aptly characterized as “sophisticated, self-propelled packages of DNA and enzymes” (Senger, 2003; Evans and Ganjam, 2011). “Differentiation” or “spermiogenesis” involves all the changes occurring within the adluminal compartment, which transform round spermatids into spermatozoa possessing an acrosome for penetration of the zona pellucida and a tail or flagellum to facilitate motility (Genuth, 2004b). Differentiation can be subdivided into the “Golgi,” “cap,” “acrosomal” and “maturation” phases, which correspond respectively to acrosomal vesicle formation; spreading of the acrosomal vesicle over the nucleus; elongation of the nucleus and cytoplasm and final assembly involving the formation of the post nuclear cap organization of the tail components (Senger, 2003; Evans and Ganjam, 2011). Following the nuclear and cytoplasmic reorganization which characterizes the changes to germ cells during spermiogenesis, differentiated spermatozoa are released from Sertoli cells into the lumen of the seminiferous tubules by a process referred to as “spermiation.” The complex signaling pathways and genomic imprinting involved in regulating the differentiation of round spermatids into spermatozoa are potential targets for EDCs.

#### *The cycle of the seminiferous epithelium*

In most sexually mature mammals, spermatozoa are produced continuously, with the entry of germ cells into the proliferation phase of spermatogenesis occurring in a coordinated cyclic manner (Genuth, 2004b). Spermatogonia A in a given region of the seminiferous tubule commit to proliferate in a synchronous manner, with cohorts of their progeny germ cells (cellular generations) connected by intercellular bridges and developing and differentiating in unison (Thomas and Thomas, 2001; Senger, 2003; Evans and Ganjam, 2011). Including spermatogonia A, four or five generations or concentric layers of sperm precursors are present in each cross-section of the seminiferous tubules (Figure 19.1) (Thomas and Thomas, 2001; Senger, 2003; Haschek *et al.*, 2010; Evans and Ganjam, 2011). The cycle of the seminiferous epithelium in most mammals is characterized by germ cells in each spermatogenic phase associating with contiguous generations in a repeatable pattern of specific cellular associations or “stages” (Thomas and Thomas, 2001; França *et al.*, 2005). There is generally only one

stage per seminiferous tubular cross-section in subprimates (França *et al.*, 2005), and each stage transitions into the next at predictable intervals (Senger, 2003). At any given point along a seminiferous tubule, the entire cycle of the seminiferous epithelium occurs over a set time interval closely associated with the spermatogonial turnover rate for that particular mammalian species (Thomas and Thomas, 2001; Haschek *et al.*, 2010). The number and duration of the various stages of the cycle of the seminiferous epithelium vary with species (Senger, 2003), and various classification schemes have been used, based on the morphological characteristics of the spermatid nucleus or the development of the acrosomic system (França *et al.*, 2005). In subprimates, sequential stages are arranged along the length of the seminiferous tubule in consecutive order, forming a "spermatogenic wave" (Senger, 2003; Haschek *et al.*, 2010). The progeny of one spermatogonium A will progress through approximately 4.5 cycles of the seminiferous epithelium before being released into the lumen of the seminiferous tubule and progressing through the rete testis into the excurrent duct system (Thomas and Thomas, 2001). An understanding of the cycle of the seminiferous epithelium is very useful for the evaluation of the effects of xenobiotics on spermatogenesis and for the determination of populations of germ cells most susceptible to a given toxicant.

### Male reproductive physiology

#### *Gonadal steroid synthesis in the testes*

The endocrine events which regulate spermatogenesis and sexual behavior in males are very distinct from those which take place in females (see below). The primary gonadal steroids produced by the testes are androgens (testosterone and DHT (also produced from testosterone in selected non-gonadal tissues)) and estrogens (primarily estradiol in most species), which are now recognized as playing essential roles in male reproductive development and function (O'Donnell *et al.*, 2001; Hess, 2003). Leydig cells in the interstitium synthesize pregnenolone and then progesterone from cholesterol and convert progesterone to testosterone under the influence of LH (Senger, 2003; Genuth, 2004b; Evans and Ganjam, 2011). The site of estrogen synthesis (i.e., aromatase activity) varies with the age and species of animal. In the male fetus, postnatal immature male and, in some species, the adult male, Sertoli cells within the seminiferous tubules play a major role in the aromatase-mediated conversion of testosterone to estradiol under the influence of FSH (O'Donnell *et al.*, 2001; Senger, 2003; Evans and Ganjam, 2011). In many mammals, Leydig cells in the fetal testis and, especially, the postnatal immature testis gradually begin to synthesize estrogens, and, at sexual maturity, a major portion of the estrogens in these species is produced by aromatase activity in the

Leydig cells, under the influence of LH rather than FSH (O'Donnell *et al.*, 2001; Hess, 2003; Evans and Ganjam, 2011). More recently, germ cells have been identified as another potential source of estrogens in the testis, and it is possible that germ cell-derived estrogens play major roles in regulating male reproductive function (Hess, 2003).

#### *Endocrine regulation of spermatogenesis*

While the female hypothalamus has both fully developed tonic and surge centers for GnRH release (especially prior to ovulation), the hypothalamic GnRH surge center in the male is diminished, and the anterior pituitary gland of the male does not experience surges in GnRH stimulation (Senger, 2003; Evans and Ganjam, 2011). This gender-specific alteration in the hypothalamus facilitates the normal endocrine milieu which maintains continuous spermatogenesis and stimulates normal sexual behavior (Figure 19.2). The tonic pulsatile release of GnRH induces the anterior pituitary to produce pulses of LH and FSH several times during the day and facilitates adequate LH-dependent testosterone production and, depending on the species, normal FSH-dependent Sertoli function, both of which are essential for spermatogenesis to occur continuously in the seminiferous tubules (Senger, 2003; Genuth, 2004b; Evans and Ganjam, 2011). In some species, FSH is primarily required for the onset of puberty and the initiation of spermatogenesis, with many of the functions of FSH in the immature male being taken over by testosterone in the sexually mature animal (Haschek *et al.*, 2010).

Testosterone stimulates Sertoli cells to produce several androgen-regulated proteins (including androgen-binding protein), which are required for spermatogenesis (Senger, 2003; Haschek *et al.*, 2010). Interference with this testosterone-mediated effect by antiandrogens (e.g., vinclozolin) which prevent interactions between testosterone and the androgen receptor or by the inhibition of testosterone synthesis by excessive glucocorticoids (e.g., chronic stress, alterations in endogenous glucocorticoid metabolism or the administration of xenobiotics with glucocorticoid-like activities) has the potential to adversely affect Sertoli cell function, and, therefore, spermatogenesis. Estrogens are required for various aspects of the normal development and function of Sertoli cells and germ cells within the seminiferous tubules (O'Donnell *et al.*, 2001; Hess, 2003; Evans and Ganjam, 2011). Xenobiotics which mimic or inhibit the actions of estradiol within the testis can disrupt normal spermatogenesis.

#### *Positive and negative feedback loops involved in male reproduction*

Positive and negative feedback mechanisms help maintain an endocrine environment which is conducive



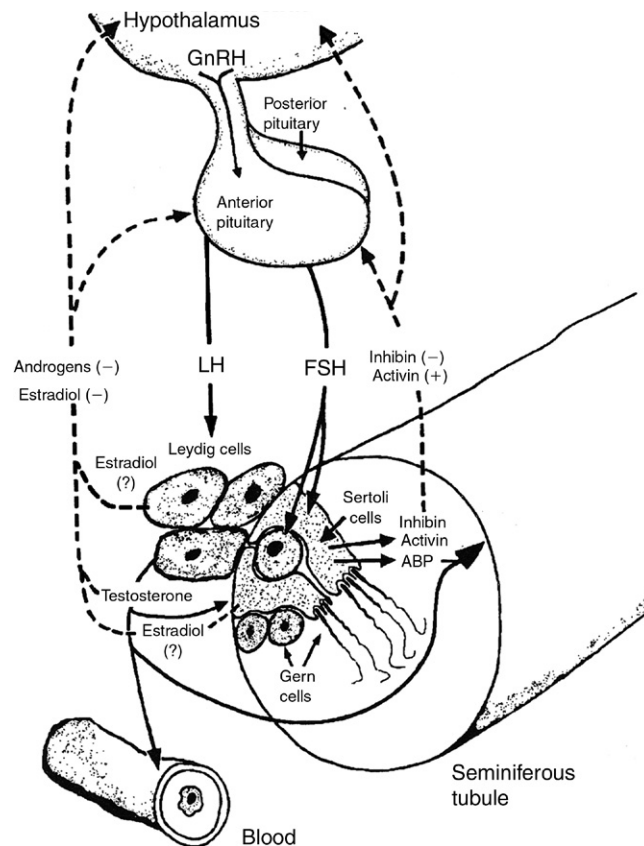
to normal male reproductive function (Figure 19.2). The Sertoli cell can produce activin and inhibin which respectively increase and decrease the secretion of FSH by gonadotropes and, in some species, GnRH release from the hypothalamus (Haschek *et al.*, 2010). Testosterone, DHT and estradiol all provide negative feedback to the hypothalamus with respect to GnRH release, and testosterone can also directly inhibit LH secretion by gonadotropes (Senger, 2003; Haschek *et al.*, 2010; Evans and Ganjam, 2011). Xenoestrogens and xenoandrogens have the potential to disturb the hypothalamic-pituitary-gonadal axis (O'Donnell *et al.*, 2001). It is currently thought that antiandrogens and a variety of other xenobiotics can interfere with these feedback loops and possibly other endocrine pathways, resulting in Leydig or interstitial cell hyperplasia (Thomas and Thomas, 2001; O'Connor *et al.*, 2002; Evans, 2011b).

#### *Epididymal and accessory sex gland function*

Epididymal development and function are dependent on the proper balance of androgenic and estrogenic stimulation and are required for normal male reproductive function and fertility. The accessory sex glands are considered to be primarily androgen dependent, and the secretions of these glands, as well as those of the epididymis, are important components of seminal fluid. Conversion of testosterone to DHT can generally occur in the epididymis, prostate and seminal vesicles. Hormonally active xenobiotics which alter the normal endocrine events associated with epididymal and accessory gland development and function can have adverse effects on male fertility.

#### *Sexual behavior, erection, emission and ejaculation*

Sexual behavior is mediated by estradiol in postnatal males and females. The conversion of the steadily produced testosterone in the male to estradiol in the brain (plus the effects of estrogens of testicular origin) results in the male being sexually receptive most of the time (Senger, 2003). Adequate libido and sexual receptivity, as well as adequate concentrations of testosterone, are necessary for erection of the penis, which is required for intromission during copulation (Sikka *et al.*, 2005). Olfactory (detection of pheromones), auditory and visual stimuli play roles in facilitating cholinergic and NANC (non-adrenergic/non-cholinergic) parasympathetic neuron-mediated penile erection, which, depending on the species, involves various degrees of nitric oxide-associated vasodilation and vascular engorgement (Senger, 2005; Sikka *et al.*, 2005). During copulation, the events which lead to emission of the secretions of the accessory sex glands and the ejaculation of spermatozoa generally involve tactile stimuli to the glans penis and stimulation by sympathetic neurons (Sikka *et al.*, 2005).



**FIGURE 19.2** The relationship between the tubular and the interstitial compartments of the testicular parenchyma and the endocrine regulation of testicular function in mammalian species is shown. Solid lines indicate positive feedback mechanisms, and dashed lines denote negative feedback pathways. The question mark associated with the production of estradiol by the Sertoli cells and Leydig cells is used to indicate that this hormone, as well as other estrogens, can be produced in the testis by either primarily Sertoli or Leydig cells, depending on the species and stage of development. Although not shown, it should be kept in mind that DHT also provides negative feedback to the hypothalamus and anterior pituitary and germ cells can also aromatize testosterone and produce estradiol. This figure was adapted, with permission, from Garner DL, Hafez ESE (2000) (modifications courtesy of Don Connor and Howard Wilson).

## **Normal female reproductive anatomy and physiology**

### *Reproductive anatomy of the female*

Although there are some distinct morphological differences between species (e.g., simplex uterus in primates, duplex cervixes in rabbits), the female reproductive tract generally consists of paired ovaries and the "tubular genitalia," which include the paired oviducts (uterine tubes) and uterine horns contiguous with a uterine body and cervix, vagina, vestibule and vulva (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). The organs involved in female reproductive function are



physiologically and morphologically dynamic and function to produce the oocyte, facilitate its fertilization, provide an environment for embryonic and fetal development, and transport the fetus from the maternal to the external environment. Variations in size, appearance, location and function of the female reproductive organs depend on the endocrine milieu dictated by the effects of sexual maturation, stage of the estrous or menstrual cycle, gestational hormone production of maternal, fetal and/or placental origin, exposure to exogenous HAAs and seasonal influences (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007).

The primary functions of the ovary are oogenesis (production of female gametes (oocytes or ova)) and steroidogenesis (production of estrogens and progesterone). The ovaries of most domestic mammals consist of a peripheral parenchymatous zone (cortex), containing various stages of follicular and luteal gland development and a central vascular zone (medulla), comprised of collagenous connective tissue rich in blood vessels (Senger, 2003; Evans and Ganjam, 2011) (Figure 19.3). The structural and functional unit of the ovary is the follicle. Follicles are classified as primordial, primary (some become atretic), secondary and tertiary (antral) follicles based on their stage of development (Evans *et al.*, 2007; Evans and Ganjam, 2011).

A primary oocyte surrounded by a single, flattened cell layer is a primordial follicle. A basal lamina separates the single layer of what will become granulosa cells from the adjacent stromal tissue which eventually develops into the theca cells (theca interna and theca externa). The granulosa cells homologous to the Sertoli cells in the testis, and the theca interna cells are the female equivalent of the Leydig cells (Senger, 2005). Following the appropriate endocrine stimulation, primordial follicles are recruited to undergo possible further differentiation into estrogen-producing antral follicles and ultimately ovulation, which results in the release of a secondary oocyte (primary oocyte in dogs) and formation of a corpus luteum (CL) which produces progesterone (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011).

### *Female reproductive physiology*

Females are born with a finite pool of primordial follicles (up to hundreds of thousands), and reproductive cyclicity (i.e., estrous or menstrual cycles) provides females with repeated opportunities for the establishment of pregnancy. The majority of mammalian species (subprimates) have estrous cycles, which reflect the physiological changes occurring between successive ovulations and/or periods of sexual receptivity (estrus) (Senger, 2005; Evans and Ganjam, 2011). Humans and non-human primates experience menstrual rather than estrous cycles and do not have defined periods of sexual

receptivity (i.e., estrus). Unlike the estrous cycles in subprimates, the reproductive cycle in menstruating animals is divided into phases (i.e., menses, proliferative and secretory phases), which are defined based on the physiological state of the uterine endometrium, rather than on the predominant ovarian structures (Senger, 2003; Genuth, 2004b; Evans and Ganjam, 2011).

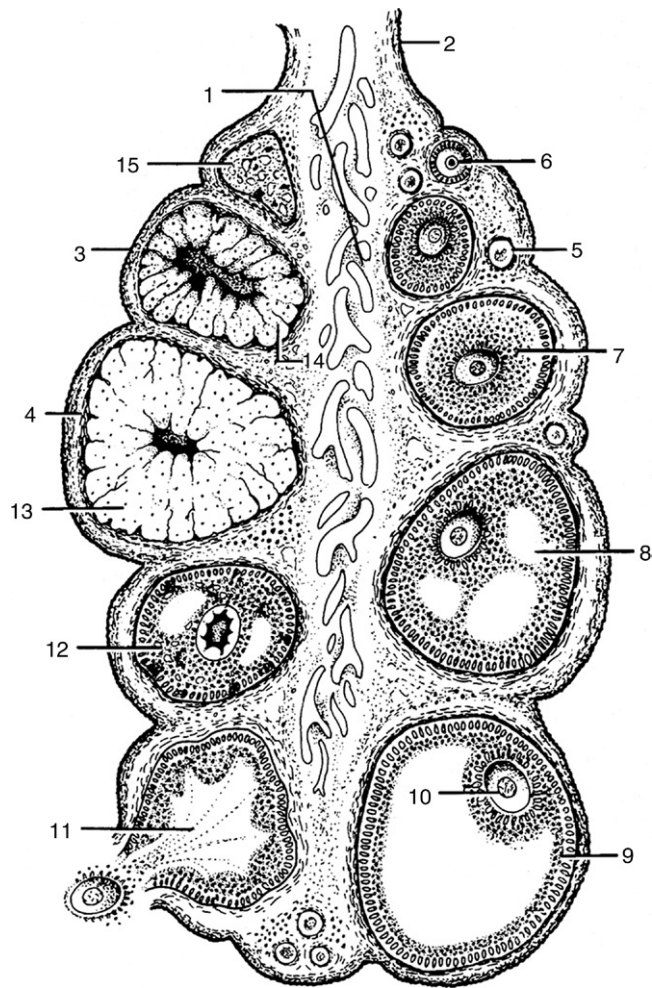
### *The estrous cycle*

The follicular and luteal phases of the estrous cycle describe the predominant ovarian structures and the corresponding gonadal steroid concentrations which result from the follicular secretion of estrogens or the luteal secretion of progesterone, respectively (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). Both the follicular and luteal phases can generally be further subdivided into two stages each, proestrus and estrus (sexual receptivity) for the follicular phase and metestrus and diestrus (sexual non-receptivity) for the luteal phase (Senger, 2003). Proestrus represents the period of transition from the diestrus dominance of progesterone to the dominance of estrogens during estrus, while metestrus represents the opposite shift in the endocrine milieu (estrogen dominance to progesterone dominance) (Senger, 2003; Evans *et al.*, 2007).

The durations of the various stages of the estrous cycle vary with species and can, depending on the animal in question, either occur throughout the year, multiple times within an ovulatory season that is dependent on photoperiod (long-day or short-day polyestrous animals) or only once a year (Senger, 2003). The domestic bitch does not have a metestrus and, in fact, is actually most receptive to copulation when estradiol is declining and there is a unique, preovulatory surge in progesterone. This endocrine environment predisposes the bitch to cystic endometrial hyperplasia and pyometra following exposure to some xenoestrogens and progestagens. Felids, which are induced (reflex) ovulators, like ferrets, mink, camelids and rabbits, the period of time following an estrus in which copulation has not occurred has been described as post-estrus rather than metestrus because there is no increase in progesterone secretion following the end of sexual receptivity (Senger, 2003). Anestrus is the time period during which reproductive cyclicity ceases and can be seasonal (estradiol and progesterone production are at basal levels) or can be associated with various endocrine milieus related to species of animal, pregnancy, lactation, stress and/or pathological conditions, some of which can be induced by xenobiotics.

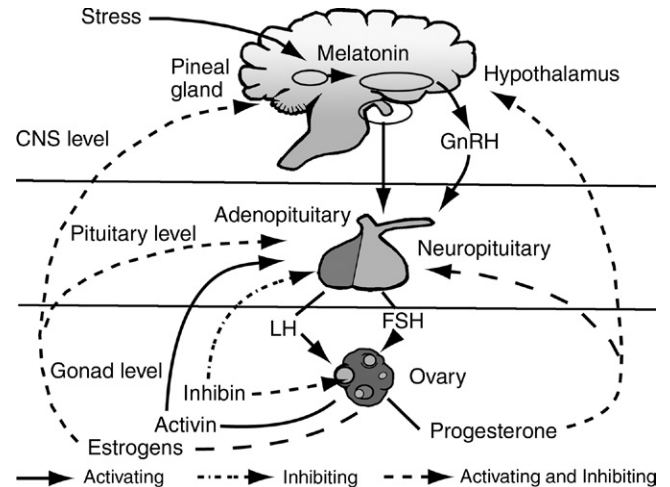
### *Follicular development*

The general sequence of endocrine and morphological changes occurring during the estrous cycle involves a variety of positive and negative feedback loops affecting



**FIGURE 19.3** Although there is some interspecies variation with respect to ovarian structure, a schematic representation of a “typical” mammalian ovary is shown to demonstrate the major ovarian structures: (1) medulla; (2) mesovarium; (3) surface epithelium; (4) tunica albuginea, which is poorly developed in the ovary as compared to the testis; (5) primordial follicle; (6) primary follicle; (7) secondary follicle; (8) early tertiary or antral follicle; (9) mature antral follicle; (10) oocyte; (11) ruptured follicle and ovulated secondary oocytes (except for the dog); (12) atretic follicle; (13) CL; (14) atretic CL; (15) corpus albicans. This figure was adapted, with permission, from [Dyce et al. \(2002\)](#) (modifications courtesy of Don Connor and Howard Wilson).

the hypothalamic–pituitary–gonadal axis and leads to the development of antral follicles, the primary source of estrogens, and, eventually, the formation of corpora lutea, which produce progesterone ([Figures 19.3 and 19.4](#)). During the time of year when females are exhibiting reproductive cyclicity, there are cyclic alterations in the pattern of hypothalamic GnRH secretion from the tonic and surge centers, which interact with the anterior pituitary to influence the relative amounts of FSH and LH secreted by anterior pituitary gonadotropes. Over the course of the ovulatory season, many (up to several hundred or more) primordial follicles leave the reserve pool



**FIGURE 19.4** The endocrine regulation of ovarian function and the feedback loops for the hypothalamic–pituitary–gonadal (ovarian) axis in the female are depicted. This figure was adapted, with permission, from [Wilker and Ellington \(2006\)](#) (modifications courtesy of Don Connor and Howard Wilson).

in a cyclic fashion (under the influence of FSH) and enter the active pool of follicles (primary follicles) undergoing growth and differentiation (folliculogenesis) and eventually atresia or ovulation ([Senger, 2003; Evans et al., 2007; Evans and Ganjam, 2011](#)). The oocyte in the developing follicle grows in size, the zona pellucida is formed and the granulosa cells surrounding the oocyte undergo mitosis and further differentiation ([Senger, 2003; Evans and Ganjam, 2011](#)). A primary follicle is transformed into a secondary follicle when there are several layers of granulosa cells. Preantral follicles (primary and secondary follicles) become antral (tertiary) follicles, when fluid from the granulosa cells of secondary follicles coalesces to form an antrum ([Evans et al., 2007; Evans and Ganjam, 2011](#)).

Cyclic increases in FSH concentrations facilitate recruitment antral follicles. Granulosa cells can produce activin which is thought to provide positive feedback to the anterior pituitary, further increasing gonadotropic FSH secretion ([Figure 19.4](#)) ([Senger, 2003; Wilker and Ellington, 2006](#)). Recruited antral follicles, which are gonadotropin sensitive, undergo several waves of follicular development beginning in metestrus and ending in proestrus ([Ginther, 1992; Senger, 2003; Evans et al., 2007](#)). The final wave of one or more dominant follicles, destined for ovulation, rather than atresia, produces the large amounts of estrogens typical of estrus and required for sexual receptivity and the preovulatory estrous surges in GnRH and LH secretion ([Senger, 2003](#)).

#### Ovarian follicular synthesis of estrogens

The production of estrogens (predominantly estradiol) by antral follicles is accomplished by a mechanism termed the “two-cell or two-gonadotropin model,”

which can vary somewhat between species (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). Cells from the theca interna and/or granulosa cells (depending on the species) produce progesterone from pregnenolone synthesized from cholesterol and, under the influence of relatively low concentrations of LH, theca interna cells convert this progesterone into androgens and, ultimately, testosterone (Evans *et al.*, 2007; Evans and Ganjam, 2011). In granulosa cells (reportedly theca interna cells in some species), the release of FSH from the anterior pituitary induces aromatase-mediated conversion of testosterone produced in the theca cells into estradiol (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). Stimulation of aromatase activity by xenobiotics can have an overall estrogenic effect on exposed animals (increased production of estradiol).

#### *The effects of estrogenic feedback on the hypothalamic–pituitary–gonadal axis*

Increasing concentrations of estrogens associated with estrus alter the hypothalamic GnRH secretory pattern and decrease pituitary secretion of FSH, while greatly increasing the amount of LH produced and released by the anterior pituitary gland (preovulatory LH surge) (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). Although inhibin produced by granulosa cells further decreases FSH secretion, dominant follicles surviving to estrus do not undergo atresia because of an enhanced sensitivity to basal FSH levels (Senger, 2003; Wilker and Ellington, 2006). Xenoestrogens have the potential to either imitate or inhibit these estradiol feedback mechanisms in sexually mature females, depending on the amount of estrogenic xenobiotic, the endocrine milieu at the time of the exposure and the relative binding affinity of the xenobiotic for ERs.

#### *Ovulation*

The granulosa cells in the one or more dominant estrous follicles (Graafian follicles) cease to divide shortly prior to ovulation and undergo further differentiation, with increased numbers (up-regulation) of LH receptors which will be responsive to the estrogen-induced preovulatory LH surge (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). As LH increases, granulosa cells (theca interna cells in some species) continue to convert pregnenolone to progesterone, but estradiol production decreases, resulting in a slight preovulatory decline in estradiol (Evans *et al.*, 2007; Evans and Ganjam, 2011). The preovulatory LH surge is associated with increased follicular pressure, degeneration of theca cells and weakening of the follicular wall, completion of the first meiotic division within the oocyte (end of meiotic inhibition except in dogs and foxes) and, finally, ovulation of a secondary oocyte arrested in metaphase II (Senger, 2003;

Evans *et al.*, 2007; Evans and Ganjam, 2011). In felids, ferrets, mink, camelids and rabbits, the preovulatory LH surge is induced by copulation (intromission or vaginal stimulation in most induced ovulators; seminal fluid in camelids). Toxicants which interfere with copulation or sexual contact in these species can interfere with the ovulatory process.

#### *Formation and function of a CL*

Following ovulation, a cascade of endocrine changes takes place in the female subprimate which facilitates the transition from sexual receptivity to non-receptivity. Once an ovulation occurs, blood concentrations of follicular estradiol and inhibin return to their basal levels, and granulosa cells continue their growth, differentiation and increased production and release of progesterone (luteinization) under the influence of LH (Evans *et al.*, 2007). The functional ovarian structure which eventually develops from each ovulated follicle is a CL, which is comprised of large and small luteal cells derived from the granulosa and theca interna cells (granulosa cells in horses), respectively (Senger, 2003; Evans *et al.*, 2007). In most species, luteal cells are responsive to LH and produce progesterone until, shortly before the usual end diestrus in non-pregnant animals, the CL undergoes luteolysis mediated by oxytocin-stimulated production of prostaglandins  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). While luteolysis is an intraovarian event in primates, oxytocin-stimulated endometrium produces the luteolysin ( $PGF_{2\alpha}$ ) in subprimates (Senger, 2003; Evans and Ganjam, 2011). Xenobiotics, which can cause endometritis or mimic the actions of oxytocin or  $PGF_{2\alpha}$  (e.g., endotoxin or lipopolysaccharide (LPS)) can be associated with premature luteolysis. Conversely, toxicants with the opposite oxytocin/ $PGF_{2\alpha}$ -related effects would be expected to disrupt normal reproductive cyclicity by prolonging the lifespan of the CL and causing a prolonged diestrus or pseudo-pregnancy (e.g., xenoestrogens in swine).

Species of animals can vary in the number of fertile ovulations and, therefore, CLs, which are characteristically associated with each estrous cycle. Monotocous mammalian species usually only ovulate a single secondary oocyte each estrous cycle. The ovaries of litter-bearing (polytocous) mammals generally develop multiple follicles which mature, ovulate and form functional CLs.

#### *Summary of the effects of estrogens and progesterone during the female reproductive cycle*

The endocrine changes which occur during the estrous cycle are reflected in behavior and the size, morphology, position and function of the tubular genitalia. Estrogens have multiple effects on the female



reproductive tract which include: the previously discussed interactions with the hypothalamus and anterior pituitary to alter the patterns GnRH and gonadotropin secretion, which govern follicular development and ovulation; facilitation of sexual receptivity; increased blood flow, genital swelling, leukocytosis, mucosal secretion and myometrial tone; altered tissue electrical conductivity; and initiation of the growth of endometrial and mammary glands (Senger, 2003). Like estrogens, progesterone also has several effects on the reproductive tract of the female, but the effects of progesterone generally oppose those of estrogens, favoring pregnancy maintenance and sexual non-receptivity over ovulation and appropriately timed sexual receptivity associated with estrogenic stimulation (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). Progesterone is generally associated with negative feedback to the hypothalamus and anterior pituitary gland which limits GnRH and gonadotropin secretion (Senger, 2003; Evans *et al.*, 2007; Evans and Ganjam, 2011). Sexual receptivity and myometrial contractility and tone are diminished in an endocrine environment dominated by progesterone, while mammary and endometrial gland development and secretion are promoted (Senger, 2003). Toxicants which disrupt the communication and coordination between the ovary and the other parts of the reproductive tract (e.g., xenoestrogens, xenoandrogens and anti-estrogens) will alter the appearance and function of the reproductive organs and can interfere with survival of the oocyte, embryo and/or fetus.

## Oocyte/sperm transport, normal capacitation of sperm and fertilization

### *Transport of the ovulated oocyte*

The primary reproductive organs involved in the transport of ovulated secondary oocytes (primary oocytes in the bitch) are the oviducts or uterine tubes. Each oviduct consists of an infundibulum, isthmus and ampulla, which have some distinct differences in structure, as well as function (Evans *et al.*, 2007; Evans and Ganjam, 2011). The ovulated ovum enters the funnel-like opening to the infundibulum and is transported through the isthmus to the ampulla or ampullary-isthmic junction for fertilization. Unlike spermatozoa which can generally survive for several days in the oviduct, secondary oocytes usually, depending on the species, are viable for 12–24 h (Genuth, 2004b). The appropriate endocrine environment is required for adequate oviductal entry and transport of ovulated oocytes to the site of fertilization. Delayed transport of oocytes within the uterine tubes can result in the death of ova before contact can be made with fertile spermatozoa.

### *Transport and capacitation of spermatozoa*

#### *Transport of spermatozoa*

During mammalian copulation, mature sperm stored in the caudae epididymides travel through the vas deferens and penile urethra to be ejaculated into the anterior vagina, cervix or uterine body of the female reproductive tract, depending on the species. Spermatozoa can be lost from the female reproductive tract by retrograde loss and phagocytosis by leukocytes (Senger, 2003). Contractions of the smooth muscle within the tubular genitalia (muscularis), as well as interactions involving components of the seminal fluid and luminal secretions of the female reproductive tract, facilitate the transport of sperm to the oviducts (uterine tubes) where, depending on the species, fertilization takes place in the ampulla or at the junction of the ampulla and the isthmus (ampullary-isthmic junction) (Senger, 2003; Genuth, 2004b). While sperm can be rapidly transported to the ampullary-isthmic junction or ampullae of the oviducts within minutes of natural or artificial semination, the relatively slow, sustained transport of motile sperm from reservoirs of spermatozoa in the cervix and uterotubal junctions is the primary mechanism by which the viable sperm that can participate in fertilization actually enter the oviducts (Senger, 2003). Xenobiotics which interfere with the endocrine milieu required for appropriate muscularis contractility and the cervical and uterine mucosal secretions which facilitate sperm transport (e.g., phytoestrogens) can prevent spermatozoa from getting to the site of fertilization in a timely manner.

#### *Capacitation of spermatozoa*

Spermatozoa can generally survive in the oviducts for several days following insemination. Ejaculated sperm are not competent to either bind to the zona pellucida or to undergo the acrosomal (acrosome) reaction, both of which are required for fertilization of ova by mature spermatozoa. Sperm must be capacitated in order to interact with the ovum. The capacitation process involves calcium influx and biochemical changes to the sperm plasma membrane which result in the “removal” or modification of epididymal and seminal plasma proteins and the exposure of the surface molecules required for spermatozoal binding to the zona pellucida of the ovulated secondary oocyte (Senger, 2003; Genuth, 2004b). Depending on the species and, to some extent, the site of their deposition, spermatozoa become capacitated within the cervix, uterus and/or the oviduct (Senger, 2003).

### *Fertilization*

Fertilization of secondary oocytes by capacitated sperm is a complex process involving a cascade of events



which prevents fertilization of an ovum by more than one sperm (polyspermy) and ends in the fusion of the male and female pronuclei (syngamy) (Senger, 2003; Evans and Ganjam, 2011). In the oviductal ampulla or at the ampullary–isthmic junction, the motility of capacitated sperm becomes hyperactive, facilitating the precise sequence of events which includes the following in their respective order: (1) sperm binding to the zona pellucida of the oocyte involving interactions between species-specific sperm and oocyte proteins; (2) the sperm acrosomal reaction, which results in the release of acrosomal enzymes and exposure of the equatorial segment of the sperm plasma membrane; (3) acrosomal enzyme-associated penetration of zona pellucida by a single spermatozoon; (4) fusion of the plasma membrane of the sperm at its equatorial segment with the plasma membrane of the oocyte; (5) membrane fusion-associated sperm engulfment and the oocyte cortical reaction, which prevents additional oocyte zona binding and membrane fusion (i.e., polyspermy prevention); female pronucleus formation and completion of meiosis; decondensation within the sperm nucleus and male pronucleus formation and, finally, the fusion of male and female pronuclei or syngamy which produces a zygote ready to undergo embryogenesis (Senger, 2003; Genuth, 2004b; Evans and Ganjam, 2011). From the complexity of the fertilization process, it is apparent that toxicants which result in subtle aberrations in sperm and oocyte formation and maturation can have profound effects on gamete function.

## The endocrinology of pregnancy and placentation

### *Gestational hormones*

Pregnancy begins with fertilization of the oocyte within the oviduct, followed by the first cleavage of the zygote, and terminates with parturition. Although the endocrine physiology and duration of mammalian pregnancy are very species specific and are characterized by a great deal of interspecies variation, the overall goals during the entire gestation for all pregnant mammals, their embryo(s) and, eventually, the maternal–fetal–placental unit are the same. A uterine environment conducive to embryonic and fetal development must be facilitated and the pregnancy (pregnancies in multitocous animals) must be maintained for the entire normal gestational length. The primary hormones involved in establishing the proper uterine environment and maintaining pregnancy are progesterone secreted by the maternal ovary and/or the placenta, as well as, in some species, a variety of placental progestagens. In addition, a variety of other endogenous hormones of maternal, fetal

and/or placental origin (depending on the species and gender of the offspring), including androgens, estrogens, prolactin, placental lactogen, equine and human chorionic gonadotropins (eCG and hCG, respectively) and relaxin, also have important gestational functions. Normal embryonic and fetal development require that gestational hormones, especially endogenous androgens and estrogens, be synthesized and secreted in sufficient quantities and at the appropriate time during pregnancy. The proper reproductive development of the female fetus is primarily dependent on exposure to estrogens at specific times during gestation. However, the male fetus must have appropriately timed exposure to normal amounts of both androgens and estrogens for normal development of the reproductive tract and optimal adult reproductive performance (Hess, 2003). Depending on the timing of exposure, EDCs, especially those which function as gonadal steroid receptor agonists and antagonists, can potentially interfere with normal gestational signaling and sexual differentiation.

### *Maternal recognition of pregnancy*

The embryo generally enters the uterus several days after fertilization. One of the first endocrine events which must occur in most mammalian species, other than those for which the timing of luteolysis and duration of pregnancy are very similar to one another (i.e., dogs and cats), is the prevention of luteolysis (i.e., entry into the next estrus or period of sexual receptivity) and the maintenance of luteal phase progesterone concentrations (Senger, 2003; Evans *et al.*, 2007). The mechanism for this embryo–endometrium interaction in subprimates (intraovarian event in primates), also referred to as “maternal recognition of pregnancy,” has been elucidated in several species and involves embryonic production of species-specific interferon- $\tau$  in ovine and bovine species (o-IFN- $\tau$  and b-IFN- $\tau$ , respectively), estradiol secretion by porcine embryos, intrauterine embryonic migration in equids and placental chorionic gonadotropin (hCG) in humans (Senger, 2003; Genuth, 2004b). The timing of “maternal recognition of pregnancy” is species specific and in subprimates generally corresponds to the time period spanning the normal oxytocin-mediated synthesis and release of PGF<sub>2 $\alpha$</sub>  from the endometrium, as well as transport of the luteolysin to the ovary. Xenobiotics which interfere with embryonic and, in the case of humans, placental development or those toxicants which mimic the actions of the luteolysin (e.g., endotoxin) can terminate early pregnancies. Some species of mammals, such as dogs, cats, camelids, goats, swine and rabbits, depend solely on luteal progesterone secretion for the maintenance of pregnancy (Senger, 2003). The placenta takes over progesterone-associated pregnancy maintenance in sheep at approximately 50 days

post-conception and between the 6th and 8th month of gestation in cattle (Senger, 2003). The uterofetoplacental unit of the mare begins to produce a unique assortment of progestagens classified as 5 $\alpha$ -pregnanes, beginning at about day 70 of pregnancy (Ginther, 1992; Evans *et al.*, 2007; Evans, 2011c). A number of toxicants (e.g., ergopeptine alkaloids) have been found to interfere with normal progestagen metabolism in the mare (Evans, 2011c; see Chapter 87 in this book).

### Placentation

Most mammalian species are “eutherian” and, during pregnancy, form a placenta comprised of both fetal and maternal components, which acts as an attachment between the fetal and the maternal systems, functions as a transient endocrine organ and plays essential roles in the exchange of gases, nutrients and metabolic wastes between the maternal and the fetal circulations (Ginther, 1992; Senger, 2003). The yolk sac, chorion, amnion and allantois are the extraembryonic membranes formed by the pre-attachment mammalian embryo (Senger, 2003). While the yolk sac in most mammalian species normally undergoes regression, the allantois and chorion generally fuse to form the allantochorion, and the fluid-filled amnion provides a shock absorbing, aquatic environment to facilitate fetal development and transport (Ginther, 1992; Senger, 2003). The allantochorionic membrane is the fetal contribution to the placenta and the chorionic villi are the structures which interdigitate with the maternal endometrium (Senger, 2003).

### Types of placentas

Mammalian placentation can be classified according to the degree of intimacy between the maternal and the fetal circulations (i.e., the number of tissue layers separating maternal and fetal blood) and by the pattern of distribution of the chorionic villi on the surface of the placenta facing the maternal endometrium (Senger, 2003). Epitheliochorial placentas (placentae) have a total of six layers separating the maternal and fetal circulations and are observed in a variety of species, including equids and swine. Ruminant placentation is described as syndesmochorial because of the transient erosion and regrowth of the maternal epithelium, which results in the intermittent exposure of maternal endothelium (capillaries) to chorionic epithelium (Senger, 2003). Canine and feline placentas are classified as endotheliochorial, and the hemochorial placentation observed in rodents and primates has essentially only chorionic epithelium separating the maternal blood from that of the fetus. The placenta of each species is associated with a typical distribution of the chorionic villi, classified as being either diffuse (e.g., equids and swine), cotyledonary (e.g., ruminants), zonary (e.g., dogs and cats) or discoid

(e.g., rodents and primates). Some species, such as the rabbit, have variations in their placentation over the course of gestation, and what begins as an epitheliochorial placenta has transformed into a hemoendothelial type of placentation by the end of pregnancy (Lehman-McKeeman, 2008).

### Placental function

In multitocous species, each fetus has its own placenta, with the previously described endocrine functions, which “attaches” the fetus to the endometrium and facilitates the exchange of gases, nutrients, metabolic wastes and xenobiotics between the fetal and the maternal circulations. Although the term “implantation” is frequently used to describe the appropriately timed attachment of the placental membranes to the endometrium, only the conceptuses of rodent and primate species undergo true implantation (Senger, 2003). Placental exchange involves the processes of simple (passive) diffusion, facilitated diffusion and active transport, as well as pinocytosis and phagocytosis of some nutrients (Senger, 2003).

The passage of materials across the placenta has been traditionally thought of as primarily a function of the intimacy (i.e., number of tissues layers) between the maternal and the fetal circulations, especially with respect to maternal immunoglobulins which cross hemo- and endotheliochorial placentas but not those types of placentae having more layers. However, since most xenobiotics cross the placenta by simple diffusion, it is currently thought that molecular size and solubility are the most important determinants of the ability of potential teratogens in the maternal circulation to cross the placenta into the fetal circulation (Senger, 2003; Lehman-McKeeman, 2008). Some toxic xenobiotics can be actively transported by mechanisms intended for structurally similar endogenous molecules (Lehman-McKeeman, 2008), and it is thought that transplacental transport of lead can mimic that of calcium (Evans *et al.*, 2003).

### The “placental barrier”

Because the placenta “blocks” the ability of very large molecules to cross from the maternal circulation into the fetal circulation, the term “placental barrier” has been used to describe this protective function of the placenta. Given that a large number of potential toxicants diffuse across the placenta and reach the fetus, the term “barrier” might be somewhat of a misnomer. However, multidrug resistance protein and enzymes involved in biotransformation of xenobiotics have been found in the placenta (Lehman-McKeeman, 2008). The placenta is multifunctional, and regardless of its relative inefficacy as a “barrier” between the maternal and fetal circulations, with respect to at least some xenobiotics, the nutritional and endocrine functions of the placenta are

essential for successful completion of the mammalian pregnancies. Placental toxicity has been discussed in much greater detail in Chapter 20 of this book.

## Normal embryonic and fetal development

### *Blastocyst formation and differentiation of the germ cell layers*

In order for a zygote to develop into a viable offspring, multiple steps involving cellular division, migration, differentiation and organization must take place. Embryonic and fetal survival requires that these various steps take place in a precise order and at set times during the gestation of each species. Within 24h following fertilization, the zygote located in the oviduct begins to divide, within the confines of the zona pellucida, into multiple blastomeres, which ultimately form a ball of cells referred to as the morula (Ginther, 1992; Senger, 2003). A fluid-filled cavity (blastocoele) develops, and the newly formed blastocyst, which is divided into cells forming either the inner cell mass (future embryo proper) or the trophoblast (future chorion), enters the uterus (Senger, 2003). The blastocyst undergoes rapid growth and “hatches” from the zona pellucida. The subsequent cellular division and differentiation results in the formation of the three germ layers (i.e., endoderm, mesoderm and ectoderm), which are destined to develop into the embryonic tissues forming the various organs and body systems, as well as the extraembryonic membranes involved in placental formation and attachment (Ginther, 1992; Senger, 2003). Germ layer differentiation leads to organogenesis and the transformation of an embryo into the fetus which continues to grow and develop for the remainder of pregnancy. With respect to reproductive toxicity in non-rodent mammals, the organogenic and other developmental processes occurring during the first trimester of pregnancy are especially susceptible to the teratogenic effects of xenobiotics. The abnormalities induced by a teratogen are dependent on the specific developmental processes or signaling pathways targeted by that toxicant and the timing of the exposure.

### *Sex determination and sexual differentiation of reproductive function*

#### *Genotypic sex and development of the primitive sex cords*

The genotypic sex of a mammalian conceptus is determined at fertilization by the sex chromosome (X or Y) contributed by the sperm, which, in combination with the X chromosome in the ovum, denotes either a genotypically female (XX) or a male (XY) zygote. During early gestation in most species, the primordial germ cells arise from the epithelium of the embryonic yolk sac

and migrate through the developing mesentery to the gonadal (genital) ridge (testicular or ovarian anlage) in its position contiguous with the mesonephros (Senger, 2003; Evans *et al.*, 2007). Germ cells and stimulated somatic cells proliferate and organize into primitive sex cords within undifferentiated (bipotential) gonads, which have the potential to develop into either ovaries or testes (Senger, 2003; Basrur, 2006).

#### *Gonadal sex determination and phenotypic sexual differentiation*

Development of a phenotypically male or female mammalian fetus occurs during the first trimester of pregnancy in most species and consists of the determination of gonadal sex followed by the further development and differentiation of either the mesonephric or the paramesonephric ducts and regression of the other duct system. The selection of the mesonephric or paramesonephric ducts for retention and further differentiation results in the formation of genitalia (phenotypic sex) appropriate for either the male or female gonads, respectively (Genuth, 2004b). Gonadal sex determination and phenotypic sexual differentiation are dependent on complex and carefully timed signaling events and are extremely susceptible to disruption by xenobiotics. Toxicants which alter epigenetic programming or mimic or inhibit endogenous hormones can have potentially deleterious effects on sexual development (Basrur, 2006). Xenobiotic-induced abnormalities in phenotypic sexual differentiation can arise from defects in testicular formation, defects in androgen production and defects in androgenic action (Basrur, 2006; Hughes *et al.*, 2006). While some toxicant-induced abnormalities in sexual differentiation can be very obvious (e.g., hermaphroditism (presence of ovotestes), pseudohermaphroditism (differences in gonadal and phenotypic sex), hypospadias (feminized external genitalia; failure of urethral fold fusion) and cryptorchidism (failure of testicular descent)), other more subtle effects can be related to functional rather than structural abnormalities. In order to identify the steps in gonadal sex determination and phenotypic sexual differentiation most likely to be targeted by the effects of EDCs and other reproductive toxicants, it is important to understand how these processes are initiated within the fetus and how they impact subsequent fetal development.

For the last several decades, the model for gonadal sex determination and phenotypic sexual differentiation has been based on the premise that a “testis determining factor” (TDF) on the Y chromosome dictates that a gonad differentiates into a testis and initiates the cascade of endocrine changes (Figure 19.5) which results in a phenotypically male fetus (developed mesonephric duct system; regressed paramesonephric ducts) (Senger, 2003; Genuth, 2004b; Basrur, 2006; Evans and Ganjam, 2011). Without the

determination that the gonads will develop into testes, the “default” or “constitutive” pathway is followed and ovarian gonads are formed in association with a developed paramesonephric duct system and regressed mesonephric ducts (Senger, 2003; Genuth, 2004b; Basrur, 2006). While this model is useful to explain rather complex developmental processes, it should be kept in mind that other toxicant-susceptible mechanisms might also play a role in gonadal sex determination and sexual differentiation. It is apparent that very precise, sex-specific patterns of germ line epigenetic programming and interactions with somatic cells take place during the early stages of sexual differentiation (Anway and Skinner, 2006). Recent data have suggested that these signaling pathways are susceptible to epigenetic modifications induced by some antiandrogens (Anway *et al.*, 2005; Anway and Skinner, 2006). It has also been suggested that gonadal sex determination involves other genes on both sex and autosomal chromosomes that might be targeted by reproductive toxicants (Genuth, 2004b; Basrur, 2006).

#### *Development of the male phenotype*

Once previously undifferentiated gonads commit to testes development (TDF present), a coordinated series of endocrine-induced morphological changes take place, resulting in both a genotypically and a phenotypically male fetus (Figure 19.5a). The sequence of signaling and developmental changes, which result in male sexual differentiation, include the following: (1) Sertoli cell development and secretion of anti-Müllerian hormone (AMH) or Müllerian inhibiting substance (MIS); (2) AMH-induced regression of the paramesonephric (Müllerian) ducts and differentiation of Leydig cells capable of producing testosterone; (3) testosterone-facilitated development of the mesonephric or Wolffian ducts; (4) differentiation of the mesonephric ducts into the rete testes, efferent ductules, epididymides and ducti deferens; (5) development of primordial accessory sex glands and the formation of external genitalia from primordia and, finally, in most species (some exceptions in wildlife species); (6) testicular descent of the intra-abdominal testes into their extra-abdominal position in the scrotum, prior to or very shortly after birth (some species) (Senger, 2003; Genuth, 2004b; Basrur, 2006; Edwards *et al.*, 2006).

#### *Development of the female phenotype*

If the previously undifferentiated gonads do not commit to testes development (TDF absent), ovaries are formed and a cascade of morphological changes occurs in the absence of AMH and testosterone stimulation, resulting in a genotypically and phenotypically female fetus (Figure 19.5b). This sequence of “default” or “constitutive” morphological and endocrine alterations results in the following sequence of developmental events: (1)

regression of mesonephric (Wolffian ducts); (2) differentiation of the paramesonephric (Müllerian) ducts into the oviducts, uterine horns, uterine body, cervix and anterior vagina; (3) remodeling of the ovary into its typical parenchymal and cortical structure; (4) cortical development of primordial follicles, with primary oocytes arrested in meiosis and surrounded by future granulosa and theca interna cells; and (5) development of the caudal vagina and vulva from the urogenital sinus (external genitalia primordia) (Senger, 2003; Genuth, 2004b; Basrur, 2006; Edwards *et al.*, 2006; Evans *et al.*, 2007).

#### *Sexual differentiation of the brain*

Sex-specific endocrine patterns and the resulting gender appropriate sexual behaviors in animals are necessary for fertile copulations to occur and require that the brain also undergo prenatal (postnatal in some species) sexual differentiation. Although large amounts of estradiol defeminize the brain, alpha-fetoprotein prevents most of the endogenous estrogens in the female fetus from crossing the blood–brain barrier (Senger, 2003). The brain remains inherently female under the influence of minimal amounts of estradiol, and both the GnRH tonic and surge centers are maintained within the hypothalamus of the female fetus in this low-estradiol environment (Ford and D’Occhio, 1989; Senger, 2003). Testosterone produced by the fetal testes crosses the blood–brain barrier and is converted to estradiol within the brain, and, as a result of this estradiol synthesis, the hypothalamic GnRH surge center in the male fetus is minimized (Senger, 2003).

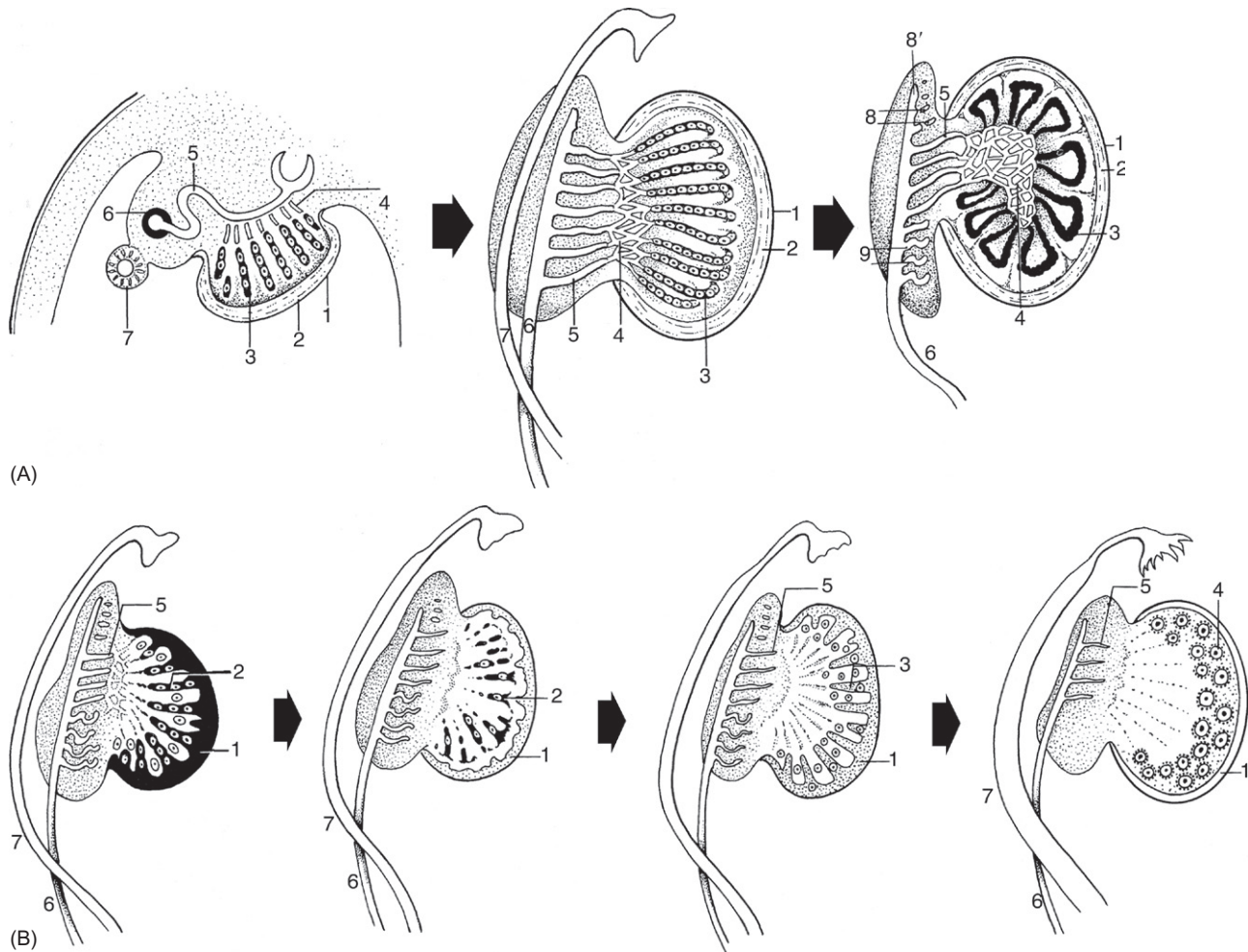
While the differentiation of male sexual behavior in large domestic animals generally involves prenatal defeminization, especially in species having longer gestations, it should be noted that postnatal defeminization of the brain is occurs in male swine and rodents (Ford and D’Occhio, 1989). There is also evidence to suggest that the males of some species with prenatal defeminization of the brain might also require postnatal exposure to androgens for maximum masculinization of the brain (Senger, 2003). Depending on the timing of exposure, xenoestrogens and exogenously administered testosterone and, possibly, some xenoandrogens, which cross the placenta and the blood–brain barrier have the potential to have profound effects on sexual differentiation of the brain and future reproductive function.

## **Parturition and lactation**

### *Physiology of parturition*

Parturition constitutes transport of the fetus and its associated membranes from the maternal to the external environment, and represents transition of the fetus to a





**FIGURE 19.5** The initial stages in the development of the testis and the formation of the excurrent duct system are shown in (A). The initial formation of the tunica albuginea isolates the epithelial cords from the surface epithelium, and the epithelial cords, rete testis and mesonephric tubules (also referred to as the mesonephric ductules or mesonephric duct system) subsequently interconnect. The epithelial cords (sex cords) will eventually become the seminiferous tubules, and the mesonephric ductules will be incorporated into the formation of the excurrent duct system. (1) Celomic epithelium; (2) tunica albuginea; (3) epithelial cords (future seminiferous tubules); (4) rete testis; (5) mesonephric tubules (later efferent ductules); (6) mesonephric duct (future epididymis (proximal portion contiguous with mesonephric tubules and ductus deferens (distal portion))); (7) paramesonephric duct; (8) cranial remnant of mesonephric duct system (aberrant ductules); (8') remnant of mesonephric duct (appendix of epididymis); and (9) caudal remnant of mesonephric duct (paradidymis). The initial stages in the development of the ovary and the formation of paramesonephric ducts are shown in (B). The epithelial cords (sex cords) penetrate and then regress within the developing ovary, eventually fragmenting and organizing into cell clusters which consist of a single oocyte surrounded by a layer of granulosa cells (primordial follicles). The paramesonephric ducts undergo further development and differentiation, and the mesonephric duct system begins to regress: (1) celomic epithelium; (2) epithelial cords which initially penetrate then regress and fragment; (3) early formation of future cortical region; (4) primordial follicles; (5) regressing mesonephric tubules; (6) mesonephric duct which will eventually regress; and (7) paramesonephric duct which will undergo further development and differentiation into the major female tubular genitalia. This figure was adapted, with permission, from [Dyce \*et al.\* \(2002\)](#) (modifications courtesy of Don Connor and Howard Wilson).

neonate. Maturation of the fetal hypothalamic–pituitary–adrenal axis plays an important role in the cascade of neural and endocrine events which lead to parturition in most mammals ([Senger, 2003](#); [Evans \*et al.\*, 2007](#)). As most clearly demonstrated in ruminants, fetal CRF stimulates the release of ACTH from the fetal pituitary, and ACTH, in turn, stimulates fetal secretion of cortisol by the adrenal glands ([Senger, 2003](#)). Elevations in fetal cortisol (fetal LH may be involved as well) activate placental

steroidogenic enzyme systems, resulting in decreased progestagens and elevated estrogens prior to parturition ([Ginther, 1992](#); [Evans \*et al.\*, 2007](#)). The resulting increase in the estrogen:progestagen ratio facilitates several important processes (e.g., cervical softening, up-regulation of myometrial oxytocin receptors, uterine synthesis of PGF<sub>2α</sub> and increased blood flow to the gravid uterus and placenta) which prepare the uterus for parturition ([Evans \*et al.\*, 2007](#)). Teratogen-induced congenital defects

in the fetal pituitary gland can result in prolonged gestation (e.g., *Veratrum californicum*), and any xenobiotic exposure causing maternal and/or fetal stress can be associated with abortion or premature parturition (e.g., nitrates and pine needle abortion).

Normal parturition approaches as neural signals caused by fetal movements and myometrial contractions, along with elevated basal levels of oxytocin and increased secretion of  $\text{PGF}_{2\alpha}$ , bring about the first stage of labor. A rapid increase in oxytocin and  $\text{PGF}_{2\alpha}$  secretion leads to rupture of the allantochorionic membrane and the commencement of the second stage of labor. Strong myometrial contractions result in the delivery of offspring, as well as the expulsion of the fetal membranes during the third stage of labor (Senger, 2003; Evans *et al.*, 2007).

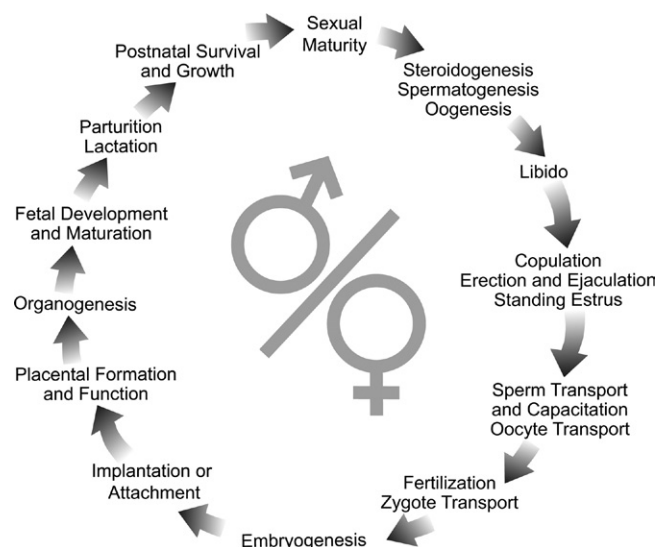
### Physiology of lactation

#### Lactogenesis

Appropriately timed lactogenesis is critical for survival of mammalian offspring. Lactogenesis is a two-stage process involving: (1) the enzymatic and cytological differentiation of the alveolar cells within the mammary gland and (2) the copious secretion of milk, which is distinct from the colostral sequestration of antibodies (Tucker, 1994). Growth hormone, aldosterone, prostaglandins, insulin, estrogens, progestagens and prolactin are required for the first stage of lactogenesis, which generally occurs during the last trimester of pregnancy (McCue, 1993; Tucker, 1994). Large increases in pulsatile prolactin secretion by lactotrobes in the anterior pituitary are necessary for the initiation of the second stage of lactogenesis, which generally occurs in close temporal association with the endocrine milieu of parturition (Evans, 1996). In many species, circulating concentrations of prolactin are elevated above basal levels for a month or two after parturition (McCue, 1993). In some species of animals, a placental lactogen performs many of the same endocrine functions as prolactin.

#### Control of prolactin secretion

Lactotropic prolactin secretion is tonically inhibited by dopamine secreted by hypothalamic neurons belonging to either the tuberoinfundibular or tuberohypophysial dopaminergic systems (TIDA and THDA, respectively) (Neill and Nagy, 1994; Evans *et al.*, 2007; Evans and Ganjam, 2011). Vasoactive intestinal peptide (VIP) and TRH are thought to act as prolactin releasing factors and can interfere with the dopamine-associated tonic inhibition of prolactin release (Evans, 1996). Oxytocin, in conjunction with the suckling reflex, will increase pituitary lactotropic production and secretion of prolactin (Neill and Nagy, 1994). In species strictly dependent on



**FIGURE 19.6** The multiple steps involved in reproductive development and function in both males and females are shown schematically to illustrate the complexity of reproduction in mammalian species and to demonstrate the various stages in the reproductive process which can be targeted for toxic insult. With respect to embryonic, fetal and placental development, it should be understood that these events are species dependent and interrelated, with many of them taking place concurrently. This figure was adapted, with permission, from Ellington and Wilker (2006) (modifications and artwork courtesy of Don Connor and Howard Wilson).

prolactin for lactogenesis (e.g., equids and swine), toxicants which mimic dopamine and tonically inhibit prolactin secretion (e.g., ergopeptine alkaloids) pose a risk to fetal survival (Evans, 2011c; see Chapter 87 in this book).

## THE MECHANISMS AND EFFECTS OF REPRODUCTIVE TOXICANTS

### The effects of EDCs on wildlife species, humans and domestic animals

It should be evident from the previous discussion that maximum reproductive efficiency, including normal embryonic and fetal development, is dependent on the structural and functional integrity of multiple organs and tissues, as well as various signaling pathways (Figure 19.6). Within the broad definition for “endocrine disruption” used in this chapter, without any restrictions as to whether the xenobiotics involved are synthetic or naturally occurring or by which specific mechanism they exert their endocrine effects, no doubt should remain in even the harshest skeptic’s mind that “endocrine disruption” is a real phenomenon. The current debate should not focus on “whether” endocrine disruption occurs, but, rather, “when” it occurs and under what

circumstances. There is increasing concern within the scientific and regulatory communities regarding the effects of prenatal and early postnatal exposures to EDCs on wildlife species and humans (Evans, 2011b). Domestic animals have been known to be susceptible to the effects of several naturally occurring reproductive toxicants in clinical settings, and it is possible that low-level environmental exposures to EDCs can also hinder reproductive function in these species (Evans, 2011a).

#### *Endocrine disruption in wildlife species*

There have been many, well-documented instances of reproductive abnormalities in species of wildlife living in environments contaminated by industrial and/or agricultural chemicals (McLachlan, 2001; Hess and Iguchi, 2002; Jobling and Tyler, 2006; McLachlan *et al.*, 2006). The deleterious reproductive effects of DDT on birds reported in Rachel Carson's *Silent Spring* have been shown to be the result of eggshell thinning related to abnormalities in prostaglandin synthesis induced by the *p,p*-DDE metabolite of DDT (Lundholm, 1997; Guillette, 2006). Wildlife populations are very likely sentinels for endocrine disruption because of the contamination of the aquatic habitats in which many of them live and the likelihood that predatory animals will have relatively high exposures to chemicals which bioaccumulate within the environment (Hess and Iguchi, 2002). Lessons learned from instances of endocrine disruption in wildlife species can be applied to EDC exposures involving humans and domestic animals (Evans, 2011b).

#### *"Androgenic" and "estrogenic" effects of EDCs on wildlife species*

Prenatal and postnatal exposures to androgenic and estrogenic environmental contaminants, as well as chemicals classified as having the opposite phenotypic effects, have been associated with various reproductive abnormalities in wildlife. Effluents from pulp and paper mills, as well as runoff from cattle feedlots where the synthetic androgen trenbolone was used for growth promotion, have been shown to be androgenic and capable of masculinizing female fish (Orlando *et al.*, 2004; Gray *et al.*, 2006). "Androgenization" or a state of indeterminate sexual development encompassing both feminization and demasculinization in males has been observed in populations of fish, amphibians, reptiles, birds and mammals and is thought to be similar to the testicular dysgenesis syndrome described in humans (Edwards *et al.*, 2006). Adult and immature amphibians exposed to the herbicide atrazine, which has been associated with increased aromatase activity in a number of species, have been reported to exhibit various manifestations of feminization (Hayes *et al.*, 2006). Hatchling, juvenile and adult male alligators (*Alligator mississippiensis*), originating

from a Florida lake previously contaminated with DDT and other persistent, bioaccumulated pesticides, as well as ethylene dibromide and DBCP, have demonstrated varying patterns of androgenization, including phallic malformations, which are thought to result from ovo exposure of maternal origin, as well as post-embryonic modifications and/or continuing environmental exposures to EDCs (Milnes *et al.*, 2006).

#### *Endocrine disruption in humans*

Based, in part, on the observations of endocrine disruption in wildlife and ongoing concerns about reproductive dysgenesis, as well as the effects of embryonic and/or fetal exposure to diethylstilbestrol (DES), the emphasis with respect to endocrine disruption in humans and one of the bases for the "Theory of Hormone Disrupting Chemicals" (THDC) or the "Environmental Endocrine Hypothesis" (Krimsky, 2000, 2001) has been the enhanced effects of prenatal, as compared to postnatal, exposures to suspected endocrine disruptors. The embryo and fetus, without a developed blood-brain barrier and with only rudimentary DNA repair mechanisms and hepatic detoxifying and metabolizing capabilities, are especially susceptible, as compared to adults, to the adverse effects of low-level exposures to xenobiotics (Newbold *et al.*, 2006). In addition, previous discussions in this chapter and other textbooks have described the important organizational events taking place during gonadal and phenotypic sexual differentiation, which are potentially very sensitive to alterations in the normal endocrine milieu (Evans, 2011b).

Although still controversial, there is a growing body of evidence to support the observation that sperm counts in men within some industrialized regions of the world have been decreasing over the last several decades (Swan *et al.*, 2000; Skakkebaek *et al.*, 2006; Jørgensen *et al.*, 2006a). In conjunction with these alterations in sperm numbers within ejaculates, there appears to have been a concurrent increase in developmental abnormalities within the male reproductive tract consistent with TDS (Skakkebaek *et al.*, 2001). Similar to what has been observed in xenobiotic-exposed wildlife, reproductive dysgenesis in human males (i.e., TDS) is associated with a suite of clinical abnormalities which include reduced semen quality, cryptorchidism, hypospadias, decreased anogenital distance and testicular cancer (Skakkebaek *et al.*, 2001; Edwards *et al.*, 2006). Failure of Sertoli cell proliferation and functional maturation within the seminiferous tubules has been one mechanism proposed for the pathogenesis of TDS (Sharpe *et al.*, 2003). The findings of a recently completely epidemiological study have suggested a relationship between decreased anogenital distance and prenatal phthalate exposure in male infants (Swan *et al.*, 2005), and a possible rodent model for



human TDS has been developed using prenatal exposure to dibutyl phthalate [di (*n*-butyl) phthalate] (Fisher *et al.*, 2003; Mahood *et al.*, 2005, 2006).

In addition to phthalates, which are used as plasticizers, a number of other widely used agricultural and industrial chemicals have been associated with adverse reproductive effects in humans and/or rodent models. In epidemiological studies a correlation has been shown between reduced semen quality in men within certain regions of the United States and the metabolites of several economically important herbicides (Swan *et al.*, 2003a, b). Metabolites of the commercially available fungicide vinclozolin have been demonstrated to interfere with interactions between androgens and their nuclear receptor, resulting in antiandrogenic effects on exposed rodents (Wong *et al.*, 1995; Monosson *et al.*, 1999; O'Connor *et al.*, 2002; Kubota *et al.*, 2003; Gray *et al.*, 2006). It has been reported that, at concentrations well below those routinely found in humans, bisphenol A, which is widely used in the plastics industry and other manufacturing processes, can initiate nongenomic estrogenic responses with plasma membrane receptors and interact with the nuclear estrogen receptor as a SERM (Welshons *et al.*, 2006). It has also recently been reported that, as might be expected, more bisphenol A is absorbed if included in the diet similar to what would be observed in actual environmental exposures, as opposed to administered as an oral bolus, which is normally what occurs in an experimental setting (Sieli *et al.*, 2011). Concerns have also been raised about the antiandrogenic activity of pyrethroid insecticides (Zhang *et al.*, 2008), which are commonly used without the awareness that these compounds have the potential to affect reproductive function at levels of exposures less than those associated with neurotoxicity. With the increased societal awareness of the possible effects of hormonally active xenobiotics on human reproduction, further research is required to make educated decisions, based on "good science," with respect to label instructions and precautions for continued use and/or regulation of economically important chemicals associated with the potential for reproductive abnormalities in humans and/or documented impairment of reproductive function in laboratory animals.

### **The effects of reproductive toxicants on domestic animals**

#### *Abortion, teratogenesis and impaired fertility in domestic animals*

Animal-based agriculture is dependent on the efficient production of viable and reproductively functional offspring. Toxicant-induced abortions, congenital defects and male or female infertility can have devastating effects on livestock production. Cattle are commonly at increased risk, especially under drought conditions, for

adult mortality and abortions in pregnant cows related to the consumption of nitrate-accumulating forages (e.g., *Sorghum* spp., oat hay (*Avena sativa*), cornstalks (*Zea mays*), and many others), which cause nitrite-induced fetal methemoglobinemia, hypoxia and, consequently, fetal stress and, potentially, even death (Casteel and Evans, 2004). Multiple congenital contractures (MCC) ("crooked calf disease") associated with the ingestion of lupines (*Lupinus* spp.) have resulted in the loss of large numbers of calves in the western United States (Panter, 2002), and multiple species of livestock exposed to swainsonine-containing plants (e.g., species of *Astragalus* and *Oxytropis* in North America species of *Swainsona* in Australia) have experienced congenital defects, abortions and/or ovarian and testicular abnormalities (Cheeke, 1998). Cleft palate, cyclops lambs, prolonged gestation and various tracheal and limb deformities have resulted from different periods of exposure of pregnant ewes to *Veratrum californicum* (false hellebore) (Burrows and Tyrl, 2001). Ergot alkaloids produced by the tall fescue endophyte *Neotyphodium coenophialum* are responsible for suboptimal reproductive performance in large numbers of cattle and horses, with late-gestational mares being particularly susceptible to endophyte-related prolonged gestation and agalactia (Evans *et al.*, 2004; see Chapters 87 and 89 of this book).

#### *Endocrine disruption in domestic animals*

With respect to the adverse reproductive effects of endocrine disruption on domestic animals, there have been many instances of impaired reproductive function involving naturally occurring EDCs of plant and fungal origin. However, there is still much to be learned about the potential adverse effects of pre- as well as postnatal environmental exposures to EDCs in these species where selection of breeding animals is often based on reproductive soundness. Postnatal exposures to phytoestrogens in some leguminous plants, including soybeans, have resulted in reproductive abnormalities and subfertility in multiple species (Cheeke, 1998; Ford *et al.*, 2006). The adverse effects of postnatal exposures to the estrogenic mycotoxin zearalenone on swine fertility have also been well documented (Cheeke, 1998). Many of the reproductive effects of the common forage-related disease syndrome referred to as "fescue toxicosis" clearly involve the endocrine disruptive effects of ergot alkaloids on prolactin secretion. In experimental studies, female swine appear to be more sensitive than rodents to the effects of the synthetic herbicide atrazine on the hypothalamic-pituitary-gonadal axis (Gojmerac *et al.*, 2004), and it is possible that companion and agricultural animals are also more susceptible than laboratory species to other EDCs. As many hormonally active xenobiotics also have important agricultural and industrial uses, it is important to continue research which attempts



to accurately predict the effects of environmental exposures to EDCs, as well as other reproductive toxicants, on domestic animals.

## Toxicants affecting the male reproductive function

There have been relatively few documented reports regarding the adverse effects of reproductive toxicants on male fertility in the major animal species of veterinary interest. Realistically the lack of examples is more likely a reflection of the limited number of controlled studies performed using non-rodent mammalian species and/or the number of toxicant-associated reproductive abnormalities which remain undiagnosed, rather than an accurate indication of the scope of the problem (Schrader, 2002). A large number of chemicals are currently thought to have the potential for causing abnormalities in male reproductive function in domestic animals. Based on extrapolations from effects observed in a various mammalian species and the limited scientific and anecdotal reports, a partial listing of these compounds is presented in Table 19.1 (Ellington and Wilker, 2006). In the following section a few selected male reproductive toxicants and their proposed mechanisms of action will be described in order to familiarize the reader with the various different ways that male fertility can be affected by reproductive toxicants.

### *Selected male reproductive toxicants and mechanisms of action*

#### *Cell-specific reproductive toxicants*

Some reproductive toxicants adversely affect specific cells within the testes. Ethane dimethane sulfonate is specifically cytotoxic to the Leydig cells, and excessive exposure to this compound results in complete loss of this population of cells within the interstitium, and, consequently, the ability of the testes to synthesize testosterone and, in some species, estrogens (Haschek *et al.*, 2010). Tri-*o*-cresyl phosphate (TOCP) is an industrial chemical used in lacquers and varnishes, which inhibits LH-induced steroidogenesis in the Leydig cells and, after Leydig cell-mediated conversion to its active metabolite, morphological abnormalities in Sertoli cells (Thomas and Thomas, 2001; Haschek *et al.*, 2010).

Sertoli cells are specifically targeted by several toxicants, including diethylhexyl phthalate (DEHP), 1,3-dinitrobenzene (DNB) and 2,5-hexanedione (metabolite of *n*-hexane) (Haschek *et al.*, 2010) and the effects of these xenobiotics are age and species specific (Thomas and Thomas, 2001). With respect to DNB, the parent compound is converted to its toxic metabolites,

nitrosonitrobenzene and nitroaniline, within the target Sertoli cells, and, similar to other Sertoli cell-specific toxicants, germ cell death and exfoliation occur secondary to toxic insult to the Sertoli cells (Haschek *et al.*, 2010). Sertoli cell microtubules appear to be the intracellular targets of 2,5-hexanedione (Thomas and Thomas, 2001; Haschek *et al.*, 2010). The fungicide dibromochloropropane (DBCP) appears to affect the Sertoli cell (Thomas and Thomas, 2001), but its metabolites epichlorhydrin and  $\alpha$ -chlorhydrin induce capillary permeability and vascular damage within the epididymis (Haschek *et al.*, 2010).

There are a number of reproductive toxicants which target-specific populations of germ cells. Spermatogonia, spermatocytes, round spermatids and elongate spermatids are specifically targeted by busulfan, 2-methoxyethanol, ethylmethane sulfonate and dibromacetic acid, respectively (Haschek *et al.*, 2010). Ionizing radiation and a variety of chemotherapeutic agents, including cyclophosphamide, nitrogen mustard, vincristine and vinblastine, generally target rapidly dividing mitotic or meiotic germ cells in the testes, and TCDD appears to adversely affect several populations of spermatozoal precursors (Thomas and Thomas, 2001). The compound 7,12-dimethylbenz[*a*]anthracene (DMBA) is toxic to spermatogonia but must undergo a stepwise biotransformation in the Leydig cell and, subsequently, the Sertoli cell to produce the ultimately toxic metabolite (Haschek *et al.*, 2010).

#### *Heavy metals*

Lead and cadmium are ubiquitous heavy metals and have both been associated with testicular toxicity and impaired fertility in a number of species. Excessive cobalt can potentially interfere with normal spermatogenesis, and severe cobalt intoxications have actually resulted in generalized hypoxia related to increased blood viscosity which affects the testes (Thomas, 1995). Chromium and vanadium have also been associated with adverse reproductive effects (Thomas and Thomas, 2001), and cis-platinum exposure has been associated with the death of spermatocytes and spermatids, as well as disruption of Sertoli cell tight junctions (Thomas, 1995). Although testicular toxicity is generally not observed with excessive parenteral exposure to zinc (other than possibly secondary to hemolytic anemia-related hypoxia) (Thomas, 1995), intratesticular injections with zinc gluconate have been successfully used for chemical castration in several species.

Divalent lead is known to interact with physiological processes involving calcium and generally has an affinity for sulfhydryl groups. Lead is reported to be directly toxic to germ cells and Leydig cells and can suppress anterior pituitary secretion of LH and FSH (Thomas and Thomas, 2001). Lead also appears to be able to adversely

TABLE 19.1 Xenobiotics/environmental and physiological factors that affect male fertility

Xenobiotic	Observed effect(s) on male
<i>Antimicrobials</i>	
Metronidazole	High doses: ↓ sperm number; ↑ abnormal morphology
Nitrofurantoin	High doses: ↓ sperm number
Tetracycline	Very high doses: ↓ sperm number; ↓ sperm capacitation; testis atrophy
Trimethoprim	1-month course: ↓ sperm number by 7–88%
<i>Antifungals</i>	
Ketoconazole	Decreased testosterone and libido; ↓ sperm number and motility
Miconazole	Interferes with testosterone in male fetus
<i>Immunosuppressants</i>	
Cyclophosphamide	Decreased sperm number; birth defects in offspring
<i>Hormones</i>	
Testosterone	Decreased sperm number; testicular degeneration
Anabolic steroids	Decreased sperm number, motility and morphology
Trenbolone	Increased sperm abnormalities
Estrogens	Decreased sperm number; behavioral feminization
Phytoestrogens	Bioaccumulation in cats on soy diets can cause poor fertility
Zearanol	Decreased spermatogenesis
<i>Antivirals</i>	
Acyclovir	Dose- and age-dependent testicular degeneration
Ganciclovir	Decreased sperm number and quality
<i>Carbonic anhydrase inhibitors</i>	
Acetazolamide	Decreased libido and impotence
<i>Psychoactive drugs</i>	
Buspirone	Decreased libido and impotence
Benzodiazepine tranquilizers	Impotence and possible ejaculatory dysfunction
Phenothiazine tranquilizers	Priapism and impotence
Tricyclic antidepressants	Decreased libido and erectile dysfunction
<i>Antihistamines</i>	
Chlorpheniramine	<i>In vitro</i> experiments: ↓ sperm motility
<i>Antineoplastics</i>	
Adrimycin	Dose- and age-dependent testicular toxicity
Cisplatin	Decreased sperm number; ↓ growth in offspring; pregnancy loss
Vincristine	Decreased sperm number; possible reversibility
<i>Antimetabolite</i>	
Cytarabine	Decreased sperm number
<i>Gastrointestinal tract drugs</i>	
Cimetidine	Decreased sperm number
Metaclopramide	Impotence
<i>Non-steroidal antiinflammatories</i>	
Naproxen	Decreased seminal prostaglandins; ↓ sperm motility
Phenylbutazone	Inhibition of sperm acrosome reaction; unknown effect on fertility
Sulfasalazine	Decreased sperm number and motility
<i>Glucocorticoids</i>	
Prednisone	Decreased sperm number and motility; ↓ testosterone
<i>Herbicides</i>	
2,4-D (dichlorophenoxyacetic acid)	Abnormal sperm quality; testicular degeneration; ↓ fertility
Diquat and paraquat	Altered sexual differentiation in the male
<i>Solvents</i>	
Nitrobenzene	Decreased sperm number and motility; testicular degeneration
Naphtha	Decreased fertility
<i>Phthalic acid esters</i>	
Diethylhexaphthalate (DEHP)	Testicular atrophy
<i>Gasoline additives</i>	
Ethylene dibromide	Testicular degeneration and poor sperm quality
<i>Insecticides</i>	
Carbamates	Decreased sperm quality
Chlorinated hydrocarbons	Testicular degeneration and atrophy
Methoxychlor	Testicular degeneration associated with estrogenic activity
Kepone	Decreased sperm number
Lindane	High doses: testicular toxicant
	In utero exposure: ↓ sperm number and ↓ testosterone in offspring

(Continued)

TABLE 19.1 (Continued)

Xenobiotic	Observed effect(s) on male
Organophosphates	No information
Pyrethrins	<i>In vitro</i> : 40–60% ↓ in testosterone binding to androgen receptor
<i>Fungicides/nematocides</i>	
DBCP (dibromochloropropane)	Decreased sperm number; testicular toxicant
<i>Fungicide</i>	
Vinclozolin	Antandrogen: disruption of male phenotypic sexual differentiation
<i>Heavy metals</i>	
Cadmium	High doses: ischemic necrosis of the testis
Chromium	Decreased testosterone; ↓ sperm number
Lead	Decreased testosterone; ↓ sperm number; ↓ fertilization rates
Mercury	Decreased sperm quality
<i>Miscellaneous xenobiotics</i>	
Dioxin	Decreased libido; abnormal sperm morphology; ↓ response to GnRH
DBP (dibutyl phthalate)	Altered sexual differentiation
PCBs	Decreased sperm number; altered hypothalamic–pituitary–adrenal axis
Gossypol	Decreased sperm number
Ethylene glycol	Decreased sperm number and motility
<i>Environmental factors</i>	<i>Observed effect(s) on male</i>
Heat	Damaged sperm chromatin and quality
Microwaves	Decreased sperm number
Radiation	High doses: death of stem cells and permanent azoospermia
Stray voltage (AC and DC)	Decreased sperm number
<i>Physiological factors</i>	<i>Observed effect(s) on male</i>
Stress	Decreased sperm motility
Fever (hyperthermia)	Damaged sperm chromatin and quality

This table was adapted, with permission, from Ellington and Wilker (2006).

affect the ability of spermatozoa to fertilize ova, but this effect, like others associated with lead exposure, appears to be dependent on age and individual variations in susceptibility, adaptation and reversibility (Sokol, 2006).

Like lead, cadmium is thought to adversely affect male reproduction by several different mechanisms. With respect to spermatogenesis, the stage of the seminiferous epithelium associated with spermiation appears to be specifically inhibited by cadmium (Thomas, 1995). Cadmium has also been shown to have possible interactions with the hypothalamic–pituitary–gonadal axis (Akinloye *et al.*, 2006). The endothelium of the testicular and epididymal vasculature is extremely susceptible to toxic insult by cadmium, potentially resulting in reduced vascular perfusion and testicular necrosis (Haschek *et al.*, 2010). Cadmium can also alter the actin filaments in the junctional complexes between adjacent Sertoli cells, thereby disrupting the integrity of the blood–testis barrier (Thomas and Thomas, 2001). Cadmium can interfere with the cellular metabolism of zinc, an essential trace element necessary for normal reproductive function, and diets deficient in zinc can predispose individuals to the toxic effects of cadmium (Akinloye *et al.*, 2006). Pre-treatment with zinc has been reported to reduce the incidence of cadmium-induced Leydig cell cytotoxicity and neoplasia (Thomas, 1995).

### Gossypol

Gossypol is a yellow, polyphenolic pigment, which is contained in most of the parts of plants belonging to the *Gossypium* genus and is concentrated in pigment glands within the seeds (Morgan, 2004; Casteel, 2007). Gossypol exists as two isomers (enantiomers) within plants (+ and the more toxic –), and these isomeric forms can be non-toxic and bound to plant proteins or toxic and “free” or unbound (Cheeke, 1998). The concentrations of the toxic free form of gossypol vary widely in whole seeds and meals, with the gossypol in direct solvent-extracted cottonseed meal being much more readily bioavailable than the gossypol contained in whole seeds (Cheeke, 1998; Casteel, 2007).

Gossypol can cause systemic and reproductive disease syndromes, depending on the species of exposed animal and the dosage of free gossypol consumed (Randel *et al.*, 1992). The toxic effects of gossypol are cumulative, and systemic disease, characterized by hepatic, renal, cardiovascular and pulmonary abnormalities, is generally observed in monogastric animals (Cheeke, 1998). Mature ruminants are considered to be relatively resistant to the severe systemic effects of free gossypol because of the propensity of this form of the pigment to become bound to proteins in ruminal fluid and, therefore, “detoxified” (Casteel, 2007). Gossypol-induced male subfertility has

been observed in monogastrics and, especially, ruminant species and is dependent on the dosage of free gossypol and the duration of gossypol exposure (Randel *et al.*, 1992; Cheeke, 1998).

Exposure of peri-pubertal or sexually mature males to sufficient dosages of free gossypol adversely affects the seminiferous epithelium and disrupts normal spermiogenesis, resulting in spermatozoa with aplastic midpieces (i.e., segmental aplasia of the mitochondrial sheath) (Randel *et al.*, 1992; Chenoweth *et al.*, 2000). Additional sperm abnormalities, possibly associated with gossypol-induced oxidative damage (Velasquez-Pereira *et al.*, 1998), can potentially develop as stressors related to the acquisition of motility in the epididymidis, and alter the structural integrity of already weakened spermatozoa (Chenoweth *et al.*, 2000). The spermatozoal abnormalities induced by exposure of immature bulls to free gossypol are most likely reversible (Hassan *et al.*, 2004) and can be ameliorated by concurrent treatment with vitamin E (Velasquez-Pereira *et al.*, 1998). Total dietary concentrations of free gossypol supplied as cottonseed meal or whole cottonseed should not exceed 150 and 600 ppm, respectively, in young developing bulls, or, similarly, 200 and 900 ppm in sexually mature animals (Morgan, 2004).

#### *Xenoestrogens and antiestrogens*

Reproductive function in sexually mature males can potentially be adversely affected by exposures to nuclear ER agonists or antagonists, as well as by estrogenic or antiestrogenic EDCs acting independently of receptor-mediated interactions (Evans, 2011b). However, it is clear from the feminizing effects of prenatal exposures to DES (McLachlan, 2001; Newbold *et al.*, 2006) and observations of androgyneization in wildlife species (Edwards *et al.*, 2006) that the male fetus is much more sensitive to the adverse effects of endocrine disruptors than male animals during the postnatal period (Hess and Iguchi, 2002). It is also important to remember that, even in males, xenobiotics which interfere with estrogenic signaling pathways can adversely affect normal reproductive development and function (O'Donnell *et al.*, 2001; Hess, 2003).

#### *Xenoandrogens and antiandrogens*

Normal phenotypic sexual differentiation of the male fetus, as well as all of the postnatal events which result in the delivery of fertile spermatozoa to the female reproductive tract, is dependent on appropriately timed androgenic stimulation of the male. It has been well recognized for quite some time that xenoandrogens (e.g., anabolic steroids and exogenous testosterone and DHT) can interfere with hypothalamic-pituitary-gonadal feedback mechanisms (Figure 19.2), resulting in decreased LH release, sperm abnormalities and testicular atrophy (Ellington and Wilker, 2006). In recent years, there has

been increasing interest in xenobiotics which can interfere with interactions between androgens and their receptors or, in some other way, disrupt androgen-dependent signaling pathways. The dicarboximide fungicides vinclozolin and procymidone and/or their metabolites inhibit the binding of androgens to nuclear androgen receptors and can demasculinize and feminize the prenatally exposed male fetus or induce important alterations in pre- or peri-pubertally exposed offspring (Monosson *et al.*, 1999; Gray *et al.*, 2006). Vinclozolin has also recently been shown to be capable, in some experimental settings, of inducing epigenetic modifications which facilitate the occurrence of transgenerational or vertically transmitted reproductive abnormalities (Anway *et al.*, 2005; Anway and Skinner, 2006). Other potential EDCs, including linuron, *p,p'*-DDE (another metabolite of DDT), prochloraz and, more recently, pyrethroid insecticides can also function as androgen receptor antagonists, with PBDEs acting as competitive inhibitors of the androgen receptor as well as androgen-induced gene expression (Gray *et al.*, 2006; Zhang, 2008; Evans, 2011b). The AhR-mediated effects of TCDD can interfere with the biosynthesis of testosterone and disrupt testosterone signal transduction pathways (Jana *et al.*, 1999; Sikka *et al.*, 2005).

#### *Phthalates*

It is recognized that phthalates, which are used as plasticizers and which are abundant within the environment, share a unique antiandrogenic mechanism, which can result in reproductive dysgenesis in male offspring. Unlike vinclozolin, phthalates are not androgen receptor antagonists, but it is also clear that they are not uterotrophic nor are they capable of inducing a persistent estrus, as would be expected with estrogenic EDCs (Gray *et al.*, 2006). Phthalates actually alter fetal Leydig cell function, resulting in decreased testosterone synthesis and down-regulated expression of insulin-like peptide-3, which is required for gubernacular cords formation (Foster, 2006; Gray *et al.*, 2006; Foster and Gray, 2008). Appropriately timed fetal exposure to di (*n*-butyl) phthalate can result in an abnormal aggregation of Leydig cells in the fetal rat testis, resulting in a failure of Sertoli cell proliferation and functional maturation, similar to what has been proposed as a possible mechanism the development of TDS in humans (Sharpe *et al.*, 2003; Mahood *et al.*, 2005, 2006).

### **Toxicants affecting the female reproductive function**

There have been many documented reports of female reproductive abnormalities associated with exposures of domestic animal species to naturally occurring EDCs



(i.e., phytoestrogens and zearalenone) (Evans, 2011a). Likewise, the adverse endocrine disruptive effects of the ergot alkaloids produced by the tall fescue endophyte *Neotyphodium coenophialum* are also very well understood (Evans *et al.*, 2004; see Chapter 87 in this book). Unfortunately, however, there are still instances of toxicant-induced subfertility which very likely go unrecognized. An effort will be made to review some of the major mechanisms of action for toxic insult to the female reproductive tract in domestic animals, in the hope that the reader might better understand the potential scope of xenobiotic-induced reproductive effects.

### **Selected female reproductive toxicants and mechanisms of action**

#### *Cell-specific reproductive toxicants*

In general, the effects of toxicants on specific cell types within the female reproductive tract, and especially the ovaries, are not as well understood as they are in the testes (Thomas and Thomas, 2001). Many female reproductive toxicants do not target particular cell lines *per se*, but, rather, disrupt the endocrine milieu of the tubular genitalia or cause changes in ovarian structures secondary to alterations in the hypothalamic–pituitary–gonadal axis (Haschek *et al.*, 2010). There are, however, a number of xenobiotics considered to be “ovotoxic.” Phthalates and TCDD can delay or decrease ovulations (Devine and Hoyer, 2005). Ionizing radiation and some of the same chemotherapeutic agents reported to adversely affect rapidly dividing germ cells within the testes (e.g., cyclophosphamide, nitrogen mustard and vinblastine) can also adversely affect primordial follicles within the ovary (Thomas and Thomas, 2001; Devine and Hoyer, 2005). Several PAHs (i.e., BaP, 3-methylcholanthrene (3-MC) and DMBA) and 1,3-butadiene appear to target oocytes in preantral follicles, and DMBA, BaP and 1- and 2-bromopropane, as well as 1,2-dibromopropane, can adversely affect antral follicular development (Devine and Hoyer, 2005). Exposure to free gossypol has been associated with increased numbers of degenerating embryos in heifers (Casteel, 2007).

Like the testes, the ovaries also have some xenobiotic biotransformation capabilities (Thomas and Thomas, 2001; Haschek *et al.*, 2010). As in other organs, oxidative damage can adversely affect ovarian structure and function. In the case of 1,3-butadiene and 4-vinylcyclohexene, the adverse effects of these toxicants on small and growing follicles are due, in part, to the toxic actions of the epoxidated metabolites of these xenobiotics (Devine and Hoyer, 2005).

#### *Heavy metals*

The ovaries do not appear to be as sensitive to the toxic effects of heavy metals as do the testes, and those

adverse effects which are observed are more subject to variation between species than what was observed in the male gonads (Thomas, 1995). Anterior pituitary release of FSH and LH and ovarian steroidogenesis appear to be inhibited by cadmium in the female (Hoyer, 2006). With respect to lead, the neuroendocrine function of the hypothalamic–pituitary–gonadal axis appears to be targeted by lead in the female, as well as in the male (Thomas, 1995; Hoyer, 2006).

#### *Phytoestrogens*

Several genera of leguminous plants produce estrogenic compounds collectively referred to as “phytoestrogens,” which can be associated with clinically relevant effects in livestock species and companion animals. Species of clover, including subterranean clover (*Trifolium subterraneum*), red clover (*Trifolium pratense*), white clover (*Trifolium repens*) and alsike clover (*Trifolium hybridum*), contain phytoestrogens classified as isoflavones (Cheeke, 1998; Burrows and Tyrl, 2001; Mostrom and Evans, 2011; Evans, 2011a). It should also be noted that other leguminous plants, such as soybean (*Glycine max*), which is commonly used as a feed source for agricultural animals, can contain varying amounts of isoflavones. Alfalfa (*Medicago sativa*) contains another class of phytoestrogenic compounds referred to as coumestans (Cheeke, 1998; Mostrom and Evans, 2011).

The relatively inactive, “parent” glycosides for the most clinically relevant isoflavones in clover are formononetin, biochanin A and genestin, and the total concentration of these phytoestrogens can be as high as 2 to 4% (20,000–40,000 ppm) in clover (Burrows and Tyrl, 2001). Primarily within the gastrointestinal tract and, especially, the rumen, formononetin is converted into an intermediate metabolite, daidzein, and, then subsequently into the more estrogenically active phytoestrogen, equol (Cheeke, 1998; Burrows and Tyrl, 2001). Conversely, biochanin A and genestin are initially converted into genestein and then subsequently into estrogenically inactive phenolic compounds (i.e., phenolic acid and *p*-ethylphenol).

Coumestrol is the primary phytoestrogen in alfalfa. This compound has significantly greater estrogenic activity than many of the isoflavones present in other legumes, and this activity generally decreases during the drying process involved in hay production (Cheeke, 1998; Burrows and Tyrl, 2001). Coumestrol is usually only present at very low concentrations (10 to 20 ppm) in the vegetative stages of alfalfa growth, but its estrogenic activity can vary with plant maturity and from year to year (Burrows and Tyrl, 2001; Mostrom and Evans, 2011; Evans, 2011a).

A number of clinical syndromes have been associated with phytoestrogen exposure in domestic animals.

Precocious mammary development has been reported in several instances of phytoestrogen exposure and can be especially evident in dairy breeds of cattle and goats (Evans, 2011a). "Clover disease" in sheep and, to a lesser extent, cattle is associated with the consumption of the isoflavones in clover, resulting in infertility associated with abnormal estrous cycles and structural and functional changes in the cervix (Cheeke, 1998; Burrows and Tyrl, 2001). Phytoestrogen-induced alterations in ovine cervical mucus interfere with the slow, sustained transport of motile spermatozoa from their cervical reservoirs (Cheeke, 1998). Genestein can induce structural changes and, possibly, irreversible organizational abnormalities in the cervix and uterus of exposed gilts (Ford *et al.*, 2006). Coumestan exposure in cattle can be associated with various and, sometimes, seemingly conflicting clinical presentations (e.g., hyperestrogenism, nymphomania, swelling of the external genitalia, estrus suppression, inhibition of ovulation and cystic ovarian disease) (Cheeke, 1998; Casteel, 2007). It is important to remember, particularly when clinical signs are very different from what was anticipated, that phytoestrogens, like other xenoestrogens, can function also as antiestrogens through the inhibition of LH and FSH release from the anterior pituitary and by competing with endogenous estrogens for receptor sites within the tubular genitalia (Cheeke, 1998; Evans, 2011b).

#### *Zearalenone*

The estrogenic mycotoxin zearalenone is produced by *Fusarium graminearum* (formerly *Fusarium roseum*), under certain environmental and storage conditions and, sometimes, in conjunction with vomitoxin or deoxynivalenol (DON). Cereal grains associated with zearalenone production include corn, wheat, barley and oats, and some grasses in New Zealand have also been reported to have been contaminated with zearalenone (Cheeke, 1998). Swine have been shown to be particularly susceptible to the adverse effects of zearalenone, with pre-pubertal gilts being affected by concentrations of zearalenone in the feed as low as 1 to 3 ppm (Cheeke, 1998; Casteel, 2007). The increased sensitivity of pigs to the estrogenic effects of zearalenone is most likely related to the slow metabolism and enhanced enterohepatic recirculation of zearalenone noted in this particular species (Cheeke, 1998). Cattle and other ruminants can be affected by zearalenone but only at dietary concentrations much higher than those associated with clinical signs in swine (Casteel, 2007). Hyperestrogenism in pre-pubertal gilts is characterized by swelling of the vulva and mammary glands, uterine enlargement and ovarian atrophy, and testicular atrophy and preputial swelling have been observed in immature male swine (Cheeke, 1998). As with other xenoestrogens, interference with estrogenic feedback mechanisms and various ovarian abnormalities,

including follicular cysts, have been observed with excessive exposure to zearalenone. Since estrogens are luteotrophic in swine, zearalenone can be associated with prolonged luteal phases (pseudopregnancy), as well as nymphomania in cycling gilts and sows, depending on the phase of the estrous cycle at the time of exposure.

#### *Synthetic xenoestrogens and antiestrogens*

A wide range of agricultural and industrial chemicals, as well as pharmaceuticals used in birth control preparations, have estrogenic and/or antiestrogenic activities, depending on the endocrine environment, presence of endogenous estrogens and stage of development at the time of exposure. The type of tissue and physiological response being discussed, as well as the relative distribution of ER $\alpha$  and ER $\beta$  receptors, will also affect the types of endocrine effects observed in a given circumstance. As has been emphasized previously, the developing fetus is particularly susceptible to the adverse effects of estrogenic and antiestrogenic endocrine disruptors (Hess and Iguchi, 2002).

Some of the synthetic xenobiotics most commonly discussed with respect to their estrogenic and/or, in some instances, their antiestrogenic activity include DES, DDT, PCBs, bisphenol A, nonylphenol, kepone and TCDD (MacLachlan, 2001). While the overall adverse effects of xenoestrogens have already been discussed in this chapter with respect to reproductive development and endocrine disruption, there are several unique clinical aspects of exposures to these types of xenobiotics which should be addressed for completeness. Prenatal human exposures to the synthetic, non-steroidal xenoestrogen DES have been associated with feminization of the male fetus, as well the increased occurrence of clear cell adenocarcinoma of the vagina in young women (McLachlan 2001; McLachlan *et al.*, 2006; Newbold *et al.*, 2006; Rogers and Kavlock, 2008). The use of DES for mismating or pregnancy prevention in dogs has been, in some instances, associated with an increased incidence of cystic endometrial hyperplasia and pyometra. The apparent ability of the widely distributed xenoestrogen bisphenol A to cause adverse developmental effects at very low environmental concentrations and in a manner characterized by a non-monotonic (inverted U- or U-shaped) dose response has been difficult for some researchers to reproduce and remains controversial (Welshons *et al.*, 2006). However, this particular issue is worthy of further, detailed discussions regarding appropriate reproductive endpoints and necessary experimental controls, given the topic's scientific ramifications, societal relevance and potential applications to other EDCs (Evans, 2011b).

#### *Xenoandrogens*

While the emphasis in the area of endocrine disruption has traditionally been on the adverse effects of

xenoestrogen reproductive development and function, there is increasing evidence that there are also instances of endocrine disruptors having androgenic activity. The effluents from pulp and paper mills have recently been shown to be able to masculinize female fish (Gray *et al.*, 2006). In addition, the runoff from cattle feedlots, where the synthetic androgen trenbolone was used for the promotion of growth, has also been shown to have androgenic activity and is also suspected of being associated with masculinization of females in wildlife species (Orlando *et al.*, 2004).

## Teratogenesis and abortion

### *Mechanisms of actions of teratogenesis and abortion*

A large number of xenobiotics have been classified with respect to their teratogenic potential, and these are listed in Table 19.2. The ability of EDCs to interfere with phenotypic sexual differentiation in the fetus has already been extensively reviewed in this chapter. Teratogenesis can be associated with each of the following mechanisms of action: (1) excessive cell death; (2) interference with apoptosis; (3) reduced cellular proliferation rate; (4) failed interactions between cells; (5) impaired morphogenetic movements; (6) reduced synthesis of components essential for growth and development; (7) mechanical disruption; (8) and alterations in pH (Hood *et al.*, 2002; Hood, 2006). Some teratogens are capable of more than one mechanism of action. Premature parturition or abortion can be induced by any xenobiotics which cause fetal or, potentially, maternal stress and initiate the cascade of endocrine and neural signaling events which would normally lead to parturition. Any intoxication in a pregnant animal has the potential to threaten fetal survival. Toxicants which cause sudden fetal death or complete cessation of placental function or disruption of the gestational source of progestagens can also induce abortion in animals.

### *Heavy metals*

Several heavy metals have been identified as teratogens and possible abortifacients in humans and animals. The adverse effects of *in utero* lead exposure, on the developing nervous systems of both humans and laboratory animal species, have been well documented (Evans *et al.*, 2003; Rogers and Kavlock, 2008). Prenatal exposure to organotins has been associated with pregnancy loss and impaired ossification in rodents (Ema and Hirose, 2006), and organic mercury is a known developmental neurotoxicant (Golub, 2006b). Other heavy metals, including cadmium and mercury, have been associated with placental toxicity, and this topic is covered in greater detail in Chapter 20 of this book.

### *Selected plant-associated teratogens and abortifacients*

Many potentially toxic plants have been found to induce teratogenesis and/or abortion in mammals, including swainsonine-containing species of *Astragalus* and *Oxytropis* and plants associated with nitrate/nitrite intoxication. While it is not possible to extensively review all of these plants in this chapter, there are several well-documented examples of plant-induced birth defects and abortion which clearly illustrate the basic principles regarding the pathogenesis of teratogenesis and abortion in domestic animals. There are a number of references which review the adverse effects of specific plants and/or toxicants on reproduction in some detail (Burrows and Tyrl, 2001; Evans, 2011a; Panter and Stegelmeier, 2011; Panter *et al.*, 2011).

### *Veratrum californicum*

Jervanine alkaloids (e.g., cyclopamine, cycloposine and jervine) in *Veratrum californicum* (false hellebore) and, potentially, some closely related plants have been associated with the occurrence of cyclops lambs and other developmental abnormalities in sheep, as well as, less commonly, cattle and goats (Burrows and Tyrl, 2001; Panter and Stegelmeier, 2011; Panter *et al.*, 2011). The mechanism of action of the teratogenic alkaloids in species of *Veratrum* involves interference with the *sonic hedgehog* (SHH) signal transduction pathway and the inhibition of neuroepithelial cell mitosis and migration during neurulation and decreased proliferation of chondrocytes (Cheeke, 1998; Burrows and Tyrl, 2001; Panter and Stegelmeier, 2011). The specific developmental abnormalities and the precise timing of maternal exposure to *Veratrum californicum*, which results in these defects is as follows: cyclops lambs and prolonged gestation (associated with the absence of pituitary gland) from maternal exposure on days 12 to, particularly, day 14 of gestation; embryonic death from maternal exposure on gestational days 19 to 21; cleft palate from maternal exposure on gestational days 24 to 30, metacarpal and metatarsal defects (limb reductions) and tracheal cartilage defects (tracheal stenosis) from maternal exposure on gestational days 28 to 31 and 31 to 33, respectively (Burrows and Tyrl, 2001). No abnormal effects were observed with maternal exposure to *Veratrum californicum* before day 10 or after day 35 of pregnancy, and *Veratrum*-associated birth defects can generally be prevented by avoiding exposure until at least 5 weeks after breeding.

### *Multiple congenital contractures*

Species of tobacco (*Nicotiana* spp.), poison hemlock (*Conium maculatum*) and lupines (*Lupinus* spp.) have all been shown to be able to induce MCC or "crooked calf

TABLE 19.2 Safety of drugs in pregnancy

Drug	Recommendation	Comments
<i>Antimicrobial drugs</i>		
Amikacin	C	Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity or nephrotoxicity.
Ampicillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Amoxicillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Carbenicillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Cephalosporins	A	Crosses the placenta but has not been shown to be harmful to fetus.
Chloramphenicol	C	May decrease protein synthesis in fetus, particularly in bone marrow.
Ciprofloxacin	D	Do not use during pregnancy; quinolones have been associated with articular cartilage defects.
Clavulanic acid–amoxicillin (Clavamox, Beecham)	A	Crosses the placenta but has not been shown to be harmful to fetus.
Clindamycin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Cloxacillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Dicloxacillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Doxycycline	D	Tetracyclines can cause bone and teeth malformations in fetus and may cause toxicity in mother.
Enrofloxacin	D	See ciprofloxacin.
Erythromycin	A	Appears to be safe except for erythromycin estolate, which has been shown to increase the risk of hepatotoxicity in women.
Gentamicin	C	Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity or nephrotoxicity. However, specific toxicities from gentamicin have not been reported, and it may be used for a serious infection in place of a suitable alternative.
Hetacillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Kanamycin	C	Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity or nephrotoxicity.
Lincomycin	A	Crosses the placenta but has not been shown to cause problems in fetus.
Metronidazole	C	Teratogenic in laboratory animals, but there is no information for dogs and cats. It should be avoided during the first 3 weeks of pregnancy.
Neomycin	A	Not absorbed sufficiently to cause systemic effects after oral administration.
Oxacillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Oxytetracycline	D	Toxic to fetus and may increase risk of hepatitis in mother (see tetracycline).
Penicillin G (benzyl penicillin)	A	Crosses the placenta but has not been shown to be harmful to fetus.
Streptomycin	D	See gentamicin. Streptomycin is associated with higher incidence of 8th nerve toxicity than other aminoglycosides.
Sulfonamides	B	Sulfonamides cross the placenta and have produced congenital malformations in rats and mice, but problems have not been reported in dogs or cats; in people, they have caused neonatal icterus when administered near term. Avoid long-acting sulfonamides.
Tetracycline	D	Tetracyclines can cause bone and teeth malformations in fetus and may cause toxicity in mother.
Trimethoprim–sulfadiazine (Tribrissen, Coopers)	B	Manufacturer states that it is safe during pregnancy in dogs; see also trimethoprim and sulfonamides.
Trimethoprim	B	Teratogenic in rats but probably safe in other species. Folate antagonism and bone marrow depression are possible with prolonged use.
Ticarcillin	A	Crosses the placenta but has not been shown to be harmful to fetus.
Tobramycin	C	Aminoglycoside antibiotics easily cross the placenta and may cause 8th nerve toxicity or nephrotoxicity.
Tylosin	B	No information is available.
<i>Antifungal drugs</i>		
Amphotericin-B	C	There are no known teratogenic effects, but amphotericin is extremely toxic. Use only if the disease is life threatening, in absence of a suitable alternative.
Griseofulvin	D	Teratogenic in rats; causes multiple skeletal and brain malformations in cats.
Ketoconazole	B	Teratogenic and embryotoxic in rats; antiandrogenic; stillbirths have been reported in dogs.
Miconazole	A	Apparently safe if applied topically.
<i>Antiparasitic drugs</i>		
Amitraz	C	Manufacturer states that reproduction studies have not been done; no information available.
Diethylcarbamazine	A	Manufacturer states that the drug may be given to dogs throughout gestation.
Dithiazanine iodide (Dizan, TechAmerica)	B	No information is available; iodide salts may cause congenital goiter if administered for prolonged periods during pregnancy.

(Continued)



TABLE 19.2 (Continued)

Drug	Recommendation	Comments
Fenbendazole	A	Safe. Has been administered to pregnant bitches without producing adverse effects.
Dichlorvos (Task, Solvay)	B	Caution is advised when administering cholinesterase inhibitors to pregnant animals; it should not be administered to puppies or kittens, but studies in pregnant dogs and cats suggest that there are no adverse effects during pregnancy.
Ivermectin	A	Safe. Reproduction studies in dogs, cattle, horses and pigs have not shown adverse effects.
Levamisole	C	No information available.
Mebendazole	A	Safe. In reproduction studies in dogs, it was not teratogenic or embryotoxic.
Piperazine	A	Safe. No known contraindications for the use of piperazine.
Praziquantel	A	Safe. No adverse effects were seen when tested in pregnant dogs and cats.
Thiacetarsamide (Caparsolate sodium, CEVA)	C	No specific information regarding toxicity to fetus is available. It can be hepatotoxic and nephrotoxic, and heartworm adulticide should be postponed until after parturition.
Bunamidine	A	Has been administered to pregnant bitches without problems and is safe in pregnant cats. Slight interference with spermatogenesis has been seen in male dogs.
Pyrantel	A	Safe. Toxicity studies have not shown any adverse effects.
Thienium	A	Safe. Manufacturer states that except in young puppies, there are no known contraindications.
Thiabendazole	B	Thiabendazole is not teratogenic in laboratory animals, but high doses have produced toxemia in ewes.
Trichlorfon	C	Caution is advised when administering organophosphates to pregnant animals. Congenital toxicoses have been reported following administration to pregnant sows. Manufacturer states that trichlorfon should not be administered to pregnant mares, but there are no recommendations for dogs and cats.
<i>Anticancer drugs</i>		
Doxorubicin hydrochloride (Adriamycin, Adria)	C	May produce malformations in newborn or embryotoxicity.
Azathioprine	C	May produce congenital malformations but has been used in pregnant women safely. It may be a suitable alternative to other drugs when immunosuppressive therapy is required.
Chlorambucil	C	May produce malformations in newborn or embryotoxicity.
Cisplatin	C	May produce congenital malformations, embryotoxicity or nephrotoxicity.
Cyclophosphamide	C	May produce malformations in newborn or embryotoxicity.
Methotrexate	C	May produce malformations in newborn or embryotoxicity.
Vincristine	C	May produce malformations in newborn or embryotoxicity.
<i>Analgesic drugs</i>		
Acetaminophen	C	Safety not established in dogs; toxic in cats.
Aspirin	C	Embryotoxicity has been seen in laboratory animals but not in other species. Late in pregnancy, it may produce pulmonary hypertension and bleeding problems (see text).
Flunixin meglumine	C	Safety in pregnancy has not been determined.
Gold (aurothioglucose)	D	Laboratory animal studies clearly show increased congenital malformations.
Ibuprofen	C	Safety in dogs and cats not established.
Indomethacin	C	Can be toxic in adult dogs; can cause premature closure of ductus arteriosus if administered near term.
Phenylbutazone	C	Safety has not been established. Long-term use can depress bone marrow.
Salicylates	C	Embryotoxicity has been seen in laboratory animals but not in other species. Late in pregnancy, it may produce pulmonary hypertension and bleeding disorders.
<i>Anesthetic and preanesthetic drugs</i>		
Acepromazine	B	Phenothiazines should be avoided near term; they may produce neonatal CNS depression.
Atropine	B	Crosses the placenta and has been used safely but may cause fetal tachycardia.
Butorphanol	B	Safe for short-term use. Neonatal depression can be treated with naloxone.
Codeine	B	Safe for short-term use. Neonatal depression can be treated with naloxone.
Diazepam	C	See anticonvulsants.
Fentanyl	B	Safe for short-term use. Neonatal depression can be treated with naloxone.
Glycopyrrolate	B	Safe. Does not cross placenta as readily as atropine. Studies in rats and rabbits have not revealed teratogenic effects.
Halothane	C	Decreased learning ability has been reported in rats after <i>in utero</i> exposure; depression may be seen in neonates after cesarean section; excessive uterine bleeding may be seen when administered during cesarean section.
Isoflurane	B	Probably safe. Depression may be seen in neonates after cesarean section.

(Continued)

TABLE 19.2 (Continued)

Drug	Recommendation	Comments
Ketamine	B	Probably safe. Depression may be seen in puppies delivered by cesarean section; may increase intrauterine pressure and induce premature labor.
Lidocaine	A	All local anesthetics appear to be safe when used for a local nerve block or epidural anesthesia.
Meperidine	B	Opiates can produce neonatal sedation and respiratory depression, but the effects can be reversed with the administration of naloxone.
Methoxyflurane	C	Neonatal depression is seen when used for cesarean section.
Morphine	B	Opiates can produce neonatal sedation and respiratory depression, but the effects can be reversed with the administration of naloxone.
Naloxone	A	Has been shown to be safe when administered to newborns within a few minutes after birth.
Nitrous oxide	B	Probably safe. Used frequently for cesarean section without adverse effects.
Oxymorphone	B	Opiates can produce neonatal sedation and respiratory depression, but the effects can be reversed with the administration of naloxone.
Pentobarbital	D	Associated with high incidence of neonatal mortality.
Thiamylal	C	Easily crosses the placenta; all barbiturates produce respiratory depression in fetus; however, thiobarbiturates are not as toxic as pentobarbital.
Thiopental	C	Easily crosses the placenta. All barbiturates produce respiratory depression in fetus; however, thiobarbiturates are not as toxic as pentobarbital.
<i>Gastrointestinal drugs</i>		
Antacids	A	Safe. Not absorbed systemically.
Antiemetics	B	Probably safe if administered short term.
Cimetidine	B	Safety has not been established, but no reports of toxicity in humans.
Dimenhydrinate	B	Safe if used short term.
Diphenhydramine	B	Safe if used short term.
Diphenoxylate	C	Studies have reported adverse effects in laboratory animals, but no adverse effects have been reported in pregnant dogs, cats and humans.
Laxatives	B	All laxatives, except castor oil (Squibb), are considered safe if they are used short term. Castor oil causes premature uterine contractions.
Loperamide	C	Same comment as diphenoxylate.
Metoclopramide	B	Safe in laboratory animals, but no studies available for cats or dogs.
Methscopolamine	C	Safety not established.
Misoprostol	D	Synthetic prostaglandin, causes a termination of pregnancy.
Prochlorperazine	B	No reports of toxicity when administered short term.
Ranitidine	B	Safety has not been established, but no reports of toxicity were reported in humans.
Sucralfate	A	Probably safe. Not absorbed systemically.
Sulfasalazine	B	Salicylate component is not absorbed enough to produce adverse effects; sulfonamide may produce neonatal icterus when used near term (see text).
<i>Cardiovascular drugs</i>		
Atropine	B	Probably safe but may produce fetal tachycardia.
Captopril	C	Has been shown to be embryotoxic in laboratory animals and goats.
Digitalis	A	Probably safe. No adverse effects seen in humans and laboratory animals (see text).
Furosemide	B	No adverse effects have been reported.
Dopamine	B	Probably safe at therapeutic doses.
Heparin	B	Does not appear to cross placenta.
Hydralazine	B	Probably safe. There have been reports of minor toxicity in rats, but it has been administered safely to pregnant women.
Isoproterenol	C	May cause fetal tachycardia; beta-adrenergic drugs inhibit uterine contractions.
Lidocaine	B	Probably safe. May cause fetal bradycardia.
Nitroglycerin	C	No information available.
Nitroprusside	C	There is a risk of fetal cyanide toxicity with prolonged use.
Procainamide	B	Probably safe. May cause fetal bradycardia.
Propranolol	C	May cause fetal bradycardia, respiratory depression and neonatal hypoglycemia; avoid use near term.
Quinidine	B	Probably safe. May cause fetal bradycardia.
Theophylline	B	No reports of adverse effects.
Thiazide diuretics	C	May cause increased incidence of perinatal mortality.
Warfarin	D	Causes embryotoxicity and congenital malformations, neural tube defects in laboratory animals and humans.
<i>Anticonvulsant drugs</i>		
Diazepam	C	Has been associated with congenital defects in mice, rats and people.

(Continued)

TABLE 19.2 (Continued)

Drug	Recommendation	Comments
Phenobarbital	B	Has been associated with rare congenital defects and bleeding tendencies in newborn but may be safer than other anticonvulsants (see text).
Phenytoin	C	Teratogenic in rats, mice and people.
Primidone	C	Same risks as phenobarbital and has been associated with increased incidence of hepatitis in adult dogs.
Valproic acid	C	May cause congenital malformations.
<i>Muscle relaxants</i>		
Dantrolene	C	Safety not established.
Dimethyltubocurarine	B	Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses.
Gallamine	B	Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses.
Methocarbamol	C	Safety not established; manufacturer states that it should not be administered during pregnancy.
Pancuronium	B	Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses.
Succinylcholine	B	Quarternary base with negligible placental transfer; it does not affect the fetus unless administered in large doses.
<i>Endocrine drugs</i>		
Betamethasone	C	Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor and abortion in dogs (see text).
Cortisone	C	Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor and abortion in dogs (see text).
Dexamethasone	C	Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor (see text). Dexamethasone has caused abortion and fetal death in dogs.
Diethylstilberstrol (DES)	D	Malformation of male and female genitourinary systems.
Estradiolcypionate (ECP)	D	Malformation of male and female genital tracts and bone marrow depression.
Flumethasone	C	Corticosteroids have been associated with increased incidence of cleft palate and other congenital malformations, and they may induce premature labor and abortion in dogs (see text).
Mitotane ( <i>o,p'</i> -DDD)	D	Adrenocortical necrosis.
Prednisolone	C	Although prednisolone has been administered to pregnant women without adverse effects, caution is advised (see dexamethasone). Prednisolone may be used in serious diseases in absence of a suitable alternative.
Stanozolol	D	Manufacturer states that it should not be administered to pregnant dogs and cats.
Testosterone	D	Causes masculinization of female fetus.
Thyroxine	B	Does not cross placenta easily and has not been associated with any problems.
<i>Miscellaneous drugs</i>		
Ammonium chloride	B	May cause fetal acidosis; discontinue use during pregnancy.
Aspartame (Nutra Sweet)	A	No risk.
Dimethylsulfoxide (DMSO)	C	Teratogenic in laboratory animals; manufacturers state that it should not be applied to breeding animals.

A: Probably safe. Although specific studies may not have proved the safety of all drugs in dogs and cats, there are no reports of adverse effects in laboratory animals or in women.

B: Safe for use if used cautiously. Studies in laboratory animals may have uncovered some risk, but these drugs appear to be safe in dogs and cats or these drugs are safe if they are not administered when the animal is near term.

C: These drugs may have potential risks. Studies in people or laboratory animals have uncovered risks, and these drugs should be used cautiously, as a last resort when the benefit of therapy clearly outweighs the risks.

D: Contraindicated. These drugs have been shown to cause congenital malformations or embryotoxicity.

disease" in cattle and, potentially, other species exposed to their toxic principles at the appropriate stage of gestation. The condition is characterized by cleft palate and limb and spinal contractures. Pyridine alkaloids (e.g., anabasine) in tobacco, piperidine alkaloids in poison hemlock (e.g., coniine and  $\gamma$ -coniceine) and piperidine-derived and quinolizidine alkaloids in lupines

(e.g., ammodendrine and anagyrine, respectively) cause stimulation then depression (depolarizing neuromuscular blockade) of nicotinic receptors in the fetus (Cheeke, 1998; Burrows and Tyrl, 2001; Panter, 2002). This stimulation followed by neuromuscular blockade of fetal nicotinic receptors during the critical period of palate and joint development (i.e., gestational days 40 to 100

in cattle; days 30 to 60 in sheep; days 30 to 60 in swine) results in decreased fetal movement and the failure of the palate and joints to form in a normal fashion (Panter, 2002; Panter and Stegelmeier, 2011).

#### *Pine needle abortion*

Ingestion by cattle of pine needles (*Pinus ponderosa*) containing isocupressic acid (also possibly present in the vegetation of some junipers and cypresses) results in late-term abortion (Cheeke, 1998). Inhibition of the catechol estrogen-induced blockade of potential-sensitive  $\text{Ca}^{2+}$  channels by isocupressic acid leads to enhanced entry of divalent calcium into arterial smooth muscle cells supplying the gravid uterus and results in profound vasoconstriction (Casteel, 1997; Cheeke, 1998; Burrows and Tyrl, 2001; Panter and Stegelmeier, 2011; Panter *et al.*, 2011). This isocupressic acid-induced vasoconstriction can lead to at least a 50% reduction in the blood flow reaching the fetal circulation and causes fetal stress, leading to the induction of premature parturition or abortion (Cheeke, 1998; Burrows and Tyrl, 2001). Late-term abortion occurs 2–14 days following heavy consumption of green or dry pine needles and results in up to 75% of exposed cows aborting or delivering dead or weak calves (Cheeke, 1998; Burrows and Tyrl, 2001; Casteel, 2007; Panter and Stegelmeier, 2011). Dams are frequently dull and depressed, with weak uterine contractions, incomplete cervical dilation, retained placenta and frequent post-partum metritis (Cheeke, 1998).

## CONCLUDING REMARKS

Reproduction is a critical biological process, required for financially viable livestock production, as well as species survival. Toxicant-induced abortions, congenital defects and subfertility can have devastating effects on both domestic animals and wildlife species. There is growing concern within the scientific and government regulatory communities, and especially the general public, about the potential for these toxicant-induced effects in humans. The information presented in this chapter was intended to familiarize the reader with terminology and concepts pertinent to reproductive toxicity and endocrine disruption, as well as to provide an overview of reproductive development, anatomy and physiology and aspects of these processes susceptible to toxic insult by naturally occurring and synthetic xenobiotics. The information and references provided should assist readers in making informed decisions in the course of their future clinical investigations, experimental designs and interpretations of scientific literature and/or regulatory policies.

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## Placental toxicity

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### INTRODUCTION

Placental toxicology embraces the knowledge of the responses of the placenta and fetus to chemical insult. It is a fascinating subject, as it deals with three components: mother, placenta and fetus. The subject includes structural and functional changes in the placenta, placentation, implantation, embryotoxicity, fetal resorption, functional deficits, structural malformation, growth retardation and birth defects. Placental toxicology has blossomed and matured in the last several decades from the contributions of scientists around the world. It has received enormous attention from the biologists, pharmacologists, toxicologists, teratologists and regulatory agencies, since the incidence of thalidomide in the 1960s.

The placenta is a rapidly growing organ, with a limited lifespan, which interfaces two separate genomes (mother and fetus). During pregnancy, the placenta serves many functions and undergoes numerous changes, such as the production and release of hormones and enzymes, transport of nutrients and waste products, chemical information flows between mother and fetus, implantation, cellular growth and maturation, at the terminal phase of placental life, and participation in delivery (Pelkonen *et al.*, 2006). In other words, the placenta plays many roles in the development of the fetus by serving as the lung, kidney, gut and exocrine/endocrine glands. Its role in providing nutrients to the developing fetus through maternal circulation has been established for a long time. Unfortunately, some of the toxic metals, such as cadmium and lead, are also delivered to the fetus by the same mechanism as that of the nutrients. In fact, many foreign substances, including pesticides, plant alkaloids, mycotoxins and drugs, reach the fetus with very little or no resistance.

The health of the placenta is a prerequisite for the health of the fetus. In the toxicant-exposed mother, the chemical encounters the placenta before it reaches

the fetus. Therefore, chemical-induced damage in the placenta is likely to reflect in the fetus. In essence, the health of the placenta seems as important as the health of the mother or the fetus. Vulnerability of this organ to toxic effects is enormous, since it has a considerable amount of metabolic activity, and as a result the placenta can enhance the toxicity by forming metabolites of greater toxicity than the parent compounds.

Literature abounds with ample evidence that the poisons of many classes adversely affect the mother and the fetus; however, it is sparse for the placenta. Many toxicants adversely affect fetal growth or survival at doses that do not produce any toxic effects in the mother. The evidence also suggests that malformations result from specific action of chemicals, rather than as a general response to maternal illness. Another important concern that needs to be mentioned is that the mechanism involved in maternal toxicity most often differs from the mechanism involved in fetotoxicity and teratogenicity. Thus, the placenta appears to be a complex organ, as its anatomical, physiological and metabolic characteristics vary depending on the age of the placenta. This would make it important to study placentas from different stages of pregnancy. It is beyond the scope of this chapter to discuss the placental toxicity of every single chemical, instead this chapter is focused on common poisons, such as metals, pesticides, mycotoxins, tobacco, etc. that adversely affect pregnant animals, placenta and fetus.

### ROLE OF THE PLACENTA

The placenta serves pivotal roles in fetal nutrition, respiration and excretion. The placenta also provides protection to the fetus. This organ has a limited lifespan, as the umbilical cord, placenta, amnion and chorion are



expelled soon after the birth of the fetus. For decades, the view of pharmacologists, toxicologists, teratologists and biologists has been that the placenta is a rejected tissue and may not be suitable for bioassay or evaluation of the cellular/molecular mechanisms of actions of drugs and toxic chemicals. But, due to substantial progress in placental pharmacology/toxicology research, the view has changed. Currently, several methods are available to use different parts of the placenta in screening drugs and chemicals and to study the molecular mechanisms for their pharmacological and toxicological effects. Use of pregnant animals is considered the preferred model to assess the toxicological actions of chemicals. In such circumstances, it is important to study placentas from different stages of pregnancy. However, obtaining material for such studies is difficult for practical reasons. Furthermore, in assessing placental toxicity of chemicals, all physiological and anatomical variables should be taken into consideration. In addition, there are several *in vitro* pharmacological/toxicological preparations from the placenta, which include: (1) perfused single placental cotyledon; (2) villus preparation; (3) segments of umbilical, chorionic plate and villus stem arteries and veins; (4) trophoblast plasma membrane; (5) isolated receptors and transporters; and (6) the placenta as a source of stem cells. For further details refer to [Sastry \(1997\)](#), [Pelkonen \*et al.\* \(2006\)](#), [Malek and Mattison \(2010\)](#) and [Dzierzak and Robin \(2010\)](#).

## TYPES OF PLACENTA

The placentas of different species vary in shape, internal architecture and nature of the interhemal barrier. Anatomically, the placenta is a complex organ and is classified into four types: (1) hemochorial (rat, rabbit, human); (2) endotheliochorial (cat, dog); (3) syndesmochorial (ruminants); and (4) epitheliochorial (pig, horse). The placenta has also been described as zonary in the dog, bidiscoid in the monkey and multicotyledonary in the sheep. Placental thickness depends on the number of fetal and maternal cell layers. For example, the rat and rabbit have a single layer of cells, primates and humans have three layers and pigs and horses have six layers. In addition to anatomical, there are physiological and functional differences among different types of placenta. Because of technical and practical advantages, animal placentas have been used to study placental function and pharmacological and toxicological actions. However, the hemochorial placenta has been studied more extensively compared to other types. In the hemochorial placenta, the membrane separating the maternal and fetal compartments consists of three layers (syncytiotrophoblast, connective tissue and vascular fetal endothelium). These anatomical and physiological similarities and differences in placentas of

different species should be taken into consideration when studying and evaluating placental toxicity.

## METABOLISM IN PLACENTA

Both phase I reactions (oxidation, reduction and hydrolysis) and phase II reactions (conjugation of a chemical with endogenous moiety, such as glucuronic acid or sulfate) of metabolism exist in the placenta as well as the fetus. The placenta can also be active in intermediary metabolism, such as gluconeogenesis, urea and fatty acid synthesis. The capacity for metabolic processes appears to vary with species, gender and gestational stage. Carbonic anhydrase activity, a marker of placental metabolism and transfer, is greater in the pig, rat and mink than in the horse, cow and human. Xenobiotic metabolism in the placenta seems to be similar to that in maternal tissues, but the extent of the metabolic activity is usually less. However, the metabolic activity is still enough that the placental/fetal toxicity of chemicals can be significantly modified. The placenta has an abundance of drug- and xenobiotic-metabolizing enzyme systems, i.e., cytochrome P450. Cyt P450 exists in multiple forms with distinct, but generally overlapping, substrate specificities and many of the isoforms are inducible by exposure to exogenous agents ([Pasanen and Pelkonen, 1994](#)). In the placenta, several steroid hormones are formed and metabolized by Cyt P450 systems, which may participate in xenobiotic metabolism. In general, the metabolites produced by biotransformation are usually less toxic compared to the parent compound, and thereby the xenobiotic-metabolizing enzymes in the placenta protect the fetus from potentially fetotoxic drugs and chemical toxicants. It is noteworthy that the same enzyme system can also form metabolites which are more toxic than their parent compounds. For example, oxon metabolites of organophosphorus insecticides and epoxides of cyclodiene chlorinated hydrocarbons have a greater potential for fetotoxicity/teratogenicity than their parental forms. In addition, placental metabolism has the capacity to form carcinogen–DNA adducts. Compared to phase I, phase II reactions in the placenta have not been studied in detail because the placenta contains very low activities of these enzymes.

## PLACENTAL SUSCEPTIBILITY TO TOXICANTS

Since the placenta serves as an exchanger between mother and fetus for nutrients, the health of the fetus

seems to be dependent on the health of the mother and placenta. There appear to be several mechanisms by which toxicants are concentrated in the placenta and fetal tissues in greater quantities than in the maternal tissues. The placenta is highly vascularized tissue with a large surface area that comes in contact with a relatively large volume of maternal blood, which is required for normal placental function, and thereby makes the placenta vulnerable to toxicants. In addition, the placenta has several biomolecules, such as proteins, lipids, carbohydrates, nucleic acids/nucleotides, hormones and growth factors, receptors, and drug-metabolizing and many other enzymes. The placenta, being rich in proteins, bioconcentrates chemical residues by means of protein binding and releases them into the placental circulation and ultimately into the fetus. In essence, the structure and function of the placenta are so unique that its susceptibility to chemical toxicity seems far greater than that of the mother. Thus, any damage to the placenta caused by a toxic chemical is likely to reflect in the fetus.

## PLACENTAL BARRIER FOR CHEMICAL TOXICANTS

The placenta is the entry through which the fetus is exposed to most chemical poisons, and the term placental barrier appears to be a false notion. The placenta has been characterized as a lipid membrane that permits bidirectional transfer of substances between maternal and fetal compartments rather than as a barrier. In general, the placental barrier consists of the trophoblastic epithelium covering the villi, the chorionic connective tissue and the fetal capillary endothelium. Foreign chemicals are transferred through placental membranes by passive diffusion. The two most common factors that are involved in transplacental transfer of common toxicants are: (1) physicochemical properties of the chemical and (2) type of placenta. In general, any chemical with a molecular weight <1000 readily crosses the placenta, and therefore the majority of the chemical toxicants are not restricted from reaching the fetus. It is important to mention that chemical properties, such as lipophilicity, polarity and degree of ionization, can influence the placental barrier. The second factor that predominantly influences the transplacental transfer of chemical toxicants is the type of placenta. For instance, the complex multilayered placenta of higher mammals can make it more difficult for chemicals to gain access to the fetus compared to the simpler choriovitelline or chorioallantoic type of placenta (Ala-Kokko *et al.*, 2000; Pelkonen *et al.*, 2006). A number of other factors that can influence the rate and extent of transplacental transfer of toxicants include: (1) maternal/fetal chemical gradient, (2)

uterine and umbilical blood flow, (3) molecular weight of the chemical, (4) protein binding and (5) lipid solubility. These factors can also determine the time required for maternal/fetal equilibrium of a toxicant. Direct or indirect evidence for placental transfer of chemical toxicants is determined based on residue analysis of these compounds and/or their metabolites in the placenta, umbilical cord and embryo/fetus.

## FACTORS THAT MAY INFLUENCE THE PLACENTAL TOXICITY

Anatomical, physiological and metabolic characteristics vary depending on the stage of the placenta. There are three major factors that can modify the overall toxicity of a chemical during pregnancy: (1) maternal toxicity, (2) placental transfer of a chemical and (3) placental/fetal metabolism.

### Maternal toxicity

Maternal toxicity can be defined as the transitory or permanent state of alteration in maternal physiology or behavior with a potential to cause adverse effects in the offspring during embryo/fetal or postnatal development. The common factors related to maternal toxicity are as follows:

- 1 Route of drug/chemical exposure
- 2 Maternal drug/chemical distribution
- 3 Maternal drug/chemical metabolism
- 4 Uterine blood flow
- 5 pH of the blood

Although the precise mechanism by which maternal toxicity factors are responsible for fetal toxicity or teratogenesis has remained unexplained, alterations in placental function appear to be important. It seems that maternal toxicity plays a major role in adverse fetal outcome by modifying placental function.

### Placental transfer of toxicants

The factors related to placental morphology, physiology and metabolism seem to be interrelated, and with continuing change they become more complex as gestation advances. The important placental transfer factors include:

- 1 Placental blood flow
- 2 pH of the blood

- 3 Placental permeability (passive or active transport system)
- 4 Placental maturity over gestation period (size, surface area and thickness)
- 5 Interspecies variation in placental morphology
- 6 Lipid-protein content of the membranes
- 7 Placental metabolism
- 8 Plasma protein binding
- 9 Physico-chemical properties of toxicants

Plasma protein (mainly albumin) binding appears to be the important factor that can modify placental toxicity. A chemical can cross the placenta only if the chemical is in free form, but a protein-bound form can be released into free form, since the protein binding is a reversible process. Once the chemical has reached the fetus, either it remains free to produce toxic effects, or once again it can bind to proteins of blood or fetal tissues, a phenomenon described as the “sink effect.”

### Placental/Fetal metabolism

Metabolism occurs in all placental and fetal tissues, as it occurs in maternal tissues, but the capacity differs at all stages of gestation. During pregnancy, metabolism of the toxicant is complicated by two major factors: (1) the pregnancy itself, since the general metabolic activity is significantly low, which may lower the degradation of toxicants and thereby increase the toxicity, and (2) pre-exposure or simultaneous exposure to other chemicals or environmental pollutants generally results in either reduced or enhanced metabolism, and consequently alters the placental toxicity. For example, previous exposure to drug-metabolizing enzyme inducers, such as enhanced monooxygenase activity by polychlorinated biphenyls (PCBs) or similar environmental pollutants, can potentiate the toxicity of the “thioate” type of organophosphorus insecticides.

## PLACENTAL TOXICITY OF METALS

Trace minerals and heavy metals are common contaminants of the environment due to their ubiquitous presence, and thereby pose a serious threat to the environment as well as human and animal health. In excessive concentrations, these minerals and metals are known to adversely affect pregnancy and the development of the conceptus, in addition to the pregnant mother. Placental toxicity of some important metals is described here in brief. It should be noted that much of the pertinent literature mentioned here is from laboratory animals.

### Aluminum

Aluminum (Al) is poorly absorbed following either oral or inhalation exposure, and practically none following dermal exposure. Al can also be absorbed following parenteral routes. It is distributed to many organs, including the liver, lungs, bone, kidney, spleen, heart, brain and muscles. Al also transplacentally traverses and accumulates in the fetal tissues in amounts that adversely influence fetal development. Studies conducted in mice and rabbits revealed that the placenta contained 4–5 times greater Al levels than the fetal or maternal tissues (Yokel and McNamara, 1985; Cranmer *et al.*, 1986). However, the placenta of the guinea pig does not accumulate this metal. It is important to mention that the placental accumulation of Al in mice and rabbits does not preclude its accumulation in the fetal tissues. For further details on toxicokinetics of Al, refer to Yokel (1997), Domingo (2011) and the chapter on Al in this book.

Pregnancy in general enhances susceptibility to Al toxicity. Oral administration of Al is known to cause a developmental syndrome, which includes *in utero* death, delayed ossification of skeleton, malformations and growth retardation, at doses that also influence maternal weight gain. An increased incidence of resorptions occurred in female BALB/c mice treated with 41 mg/kg/day as Al chloride by gavage on GD 7–16 (Cranmer *et al.*, 1986). Gestational exposure of mice to Al lactate (83 mg/kg/day) caused increased incidences of cleft palate, dorsal hyperkyphosis and delayed parietal ossification. Skeletal changes, such as delayed ossification and hypoplastic deformed ribs, were induced in rats exposed to 38–77 mg Al/kg/day by gavage on GD 6–14 (Patermain *et al.*, 1988). The severity of the effects is highly dependent on the form of Al administered. Studies using high Al exposures by iv or ip administration report a developmental toxicity syndrome consisting of death and resorptions, skeletal and soft tissue abnormalities, low birth weights, and growth retardation in rats, mice and rabbits (Wide, 1984; Yokel, 1997). The high incidence of resorptions was markedly greater in pregnant rats treated with Al chloride at dose levels of 75 mg/kg on GD 9–13, 100 mg/kg on GD 14–18 and 200 mg/kg on GD 9–13 (Benett *et al.*, 1975). A high incidence of dead offspring was recovered from mothers treated with Al chloride at a dose level of 200 mg/kg on GD 9–13. These studies indicate that Al can cause delays in skeletal development in pups. Gross fetal abnormalities include abnormal digits, wavy ribs, missing ribs, absence of xiphoid and poor ossification (particularly in the cranial bones, lower part of the vertebral column and the long bones of the limbs). The high incidence of skeletal defects and poor ossification in fetuses of mothers treated with Al chloride suggests that Al has an

adverse influence on fetal bone formation. This is due to the fact that Al binds with phosphate, thereby reducing the amount of phosphate available for bone formation. Depletion of phosphate can also result in fetal internal hemorrhage, due to failure of the blood clotting mechanism. Interestingly, neurotoxicity and neurobehavioral changes have been noted in offspring following Al exposure of rats, mice and rabbits at doses of Al that did not produce maternal toxicity (Yokel, 1997; Bondy, 2010; Domingo, 2011). Other forms of Al, such as Al citrate and Al nitrate, can also produce similar developmental effects. Overall, developing conceptuses are more sensitive than adults to Al toxicity.

## Arsenic

Arsenic (As) occurs in many forms and is commonly found in high concentrations in water and food. Both animal and human studies have shown that As crosses the placenta. Inorganic As crosses the placental barrier and selectively accumulates in the neuroepithelium of the developing animal embryo. Uptake, distribution and metabolism of sodium arsenite (2.5 µg/kg, po) and sodium arsenate (40 µg/kg, po) were determined in pregnant mice on GD 18 (Hood *et al.*, 1987). Maximum concentrations of As in the placenta were found at 4 and 2h after administration of arsenite and arsenate, respectively. Corresponding concentrations in the fetal tissues appeared at 24 and 6h. In the fetal tissues, inorganic As can be converted to the organic form. As is completely eliminated from the placental/fetal tissues within 24h of exposure. Unlike inorganic As, organic As does not cross the placenta, instead it is stored in the placenta. In other studies conducted on hamsters, rats and mice, As ingested at higher doses has been found to be fetotoxic and teratogenic. There is evidence that sodium arsenate and sodium arsenite are embryotoxic (Chaineau *et al.*, 1990). Common deformities include hypoplasia of the prosencephalon, somite abnormalities and failure of development of limb buds and sensory placodes. In hamsters, a single iv injection of sodium arsenate (20 mg/kg) given on GD 8 resulted in 49% malformed and 84% either malformed or resorbed embryos on GD 13 (Holmberg and Ferm, 1969). Common malformations included exencephaly, encephalocele, cleft lip/palate, micro/anophthalmia and ear malformations. In rats, the spectrum of malformations induced by As is similar to that described for hamsters. Placental/fetal toxic effects of As have been more extensively described in mice than rats and hamsters. Exposure of female mice with arsenite/arsenate on GD 9 produces the highest incidence of malformed live fetuses, whereas exposure on GD 11 or 12 produces the highest rate of resorptions. The common external malformations include exencephaly, micrognathia,

exophthalmia, anophthalmia, cleft lip, hydrocephalus, micromelia, ectrodactyly, open eyes, rib defects and vertebral defects. Rats treated with a single gavage dose of 23 mg As/kg as As trioxide on day 9 of gestation had a significant increase in post-implantation loss and a decrease in viable fetuses per litter, while those treated with 15 mg/kg showed no effects (Stump *et al.*, 1999). Rats and mice are much more sensitive than hamsters to organoarsenicals, in terms of placental toxicity. But the most sensitive species is the rabbit, in which 1.5 mg As/kg/day can increase resorptions and decrease viable fetus.

Although the exact mechanism involved in placental toxicity of As is not yet explained, As is known to interact with protein sulfhydryl groups, thereby adversely affecting the activity of many enzymes, including glutamic-oxaloacetic transaminase, pyruvate oxidase, monoamine oxidase, choline oxidase, glucose oxidase, urease, oxidoreductases and kinases. As has also been shown to adversely affect a number of enzymes that are involved in mitochondrial respiration (Rogers, 1996). As inhibits succinic dehydrogenase activity and thereby uncouples oxidative phosphorylation and causes fall of ATP levels affecting virtually all cellular functions (Na<sup>+</sup>/K<sup>+</sup> balance, protein synthesis, etc.). As also exerts its toxic effects to the developing fetus by inhibiting enzymes like thioredoxine reductase, methyltransferases and enzymes of the DNA repair mechanism, disturbing the homeostasis of neurotransmitters and hormones. In addition, As causes oxidative stress. Since As is known to affect vasculature and altered placental and/or embryonal vasculature has been suggested as a mechanism leading to neural tube defects, the embryo may be sensitive to this manifestation of As toxicity. At high dose, As impairs assembly and disassembly of microtubules, thus interfering with mitotic spindle formation and embryonal cell division. As compounds also cause chromosomal aberrations, which may disrupt cell cycling. The direct toxic effects of high levels of As in the developing embryo result not from a difference in the mechanism of toxicity during development, but rather from the existence of a unique target tissue, the neuroepithelium (ATSDR, 2005). Induction of stress proteins or heat shock proteins synthesis in the embryo has been explained as a common mechanism of teratogenesis. Animal studies have also presented evidence that inorganic As may be a transplacental carcinogen.

In essence, inorganic arsenicals are much more toxic than organic arsenicals. Trivalent As is developmentally more toxic than pentavalent As. In fact, pentavalent As compounds exert their toxic actions only after their conversion to the trivalent form. Placental/fetal toxicity of As is of serious concern, especially at the levels that are maternotoxic. For further details on placental, reproductive and developmental toxicity of AS, refer to a recent publication by Flora *et al.* (2011).



## Cadmium

Cadmium (Cd) is a naturally occurring metal found in the earth's crust. Most of the Cd is produced as a byproduct during the production of other metals, such as copper, lead and zinc. The major source of Cd exposure to animals can be from industrial pollution and environmental contamination, especially water. Much of the information obtained on developmental toxicity of Cd is from laboratory animals. By now, it is well established that the placenta itself is a target organ for Cd toxicity. In low to moderate doses, Cd is sufficiently sequestered in the placenta, whereas in high doses, Cd accumulates in the placenta, which perturbs the placental transport of essential elements (such as calcium and zinc), and causes placental necrosis and fetal toxicity. Maternal exposure to Cd is known to cause ultrastructural changes in the placenta, especially trophoblast cell layer II. Common changes include lysosomal vesiculation, nuclear chromatin clumping, nucleolar alterations and mitochondrial calcification. Cd-induced placental necrosis occurs initially in trophoblast cell layer II, which follows rapidly in the remaining trophoblasts. Subcutaneous administration of Cd chloride, Cd acetate or Cd lactate to pregnant rats at a dosage of 0.04 mmol/kg between the 17th and 21st day of gestation resulted in rapid, progressive placental damage, especially in the pars fetalis (Parizek, 1964). Cd-induced necrotic changes could occur as early as within 6 h.

The placenta serves as a remarkable barrier to Cd, and thereby it minimizes fetal toxicity. Cd is known to induce the synthesis of metallothionein (MT), which is a small protein rich in sulfur-containing amino acids and commonly synthesized in maternal tissues and the placenta. MTs retain Cd in maternal tissues and the placenta, and thereby reduce Cd transport to the conceptus. The mechanism involved in developmental toxicity of Cd can be explained based on the interaction between Cd and Zn. In brief, Cd substitutes for Zn in metalloenzymes, thereby Cd interferes with Zn transfer across the placenta. Because of the high affinity of MT for Zn, MT sequesters Zn in the placenta, impeding transfer to the conceptus. Reduced uteroplacental blood flow, reduced nutrient transport and placental toxicity by Cd seem to be the major contributing factors for low fetal birth weight, fetal toxicity, malformations and death. Cd-induced impaired fetal growth is partly due to Zn deprivation, since maternal tissue and the placenta retain Zn. In addition, Cd accumulates in the fetus and produces a variety of adverse effects directly on the fetus. These adverse effects include: (1) embryotoxicity; (2) inhibition of embryonic DNA and protein synthesis; and (3) reduced calcium handling ability of trophoblasts, which is a consequence of alterations in subcellular cytosolic calcium-binding properties. Oxidative stress

and apoptosis are also suggested as mechanisms of cell damage and death.

Maternal exposure to Cd during pregnancy can result in a variety of adverse reproductive outcomes, such as maternal toxicity, placental damage/hemorrhage, impaired implantation, increased resorptions, reduced litter size, fetal growth retardation, congenital malformation in the fetuses and embryonic/fetal death. Exposure to Cd during mid- to late gestation can result in both placental toxicity (reduced blood flow and necrosis) and diminished nutrient transport across the placenta. Exposure during the late gestation can also result in fetal death, despite low levels of Cd entering the fetus (Levin and Miller, 1980; Rogers, 1996).

Cd has also been shown to induce teratogenesis in rats, mice and hamsters. Cd sulfate administered to pregnant hamsters (2 mg/kg, iv) caused high incidence of resorptions. Live fetuses usually show a high rate of malformations, including facial clefts, exencephaly, anophthalmia, limb defects and rib fusions. In rats and mice, the common malformations are club foot, dysplasia of facial bones and rear limbs, cleft palate, micrognathia, sirenomelia/amelia, delayed ossification of the sternum and ribs, microphthalmia/anophthalmia, cryptorchism, gastroschisis and palatoschisis. For further details on placental, reproductive and developmental toxicity of Cd, refer to Flora *et al.* (2011).

## Lead

The common sources of lead (Pb) exposure include Pb-based paints, batteries, crank-case oil, ceramics, shots and sinkers, calking and roofing materials, and ammunition. Plants and animals appear to bioconcentrate lead, but lead is not biomagnified in the aquatic or terrestrial food chain. Pb exposure in pregnant animals usually occurs through the oral route. It is also known that absorption of Pb increases during pregnancy. After oral absorption, Pb is distributed to most of the tissues, but it deposits mainly in the skeleton, kidney and brain (primarily gray matter). Pb is known to cross the placenta and it accumulates in the fetus. Accumulation of Pb occurs in the fetal brain owing to lack of blood-brain barrier. Lead also accumulates in the placenta in times of fetal stress. In fact, a large maternal/fetal concentration gradient exists and the placenta poses a limited transplacental barrier. In addition, a number of adverse maternal health conditions can affect the transfer of lead to the fetus and/or the retention of Pb by the mother or the fetus.

Toxicity of Pb is largely due to its capacity to mimic calcium (Ca) and substitute it in many of the fundamental cellular processes that are Ca dependent. Lead toxicity is induced by interfering with protein/hemoprotein biosynthesis and by inhibiting membrane and

mitochondrial enzymes (Eisenmann and Miller, 1996). Pb is also known to cause deficits in cholinergic, dopaminergic and glutamatergic functions. Recent evidence suggests that oxidative stress is one of the mechanisms involved in lead pathogenesis. Following *in utero* exposure, Pb can have direct effects on the developing conceptus. In fact, the developing nervous system is the most sensitive target of lead toxicity. Developmental toxic effects of Pb have been determined in experimental animals, including rats, mice, hamsters and chicks. *In utero* exposure to Pb can cause reduction in fertility and growth retardation. Embryotoxic and fetotoxic effects of Pb seem to be dependent on the exposure on GD 9; teratogenic effects are observed with few resorptions, compared with exposure on GD 16, with hydrocephalus and CNS hemorrhage (McClain and Becker, 1975). At maternotoxic doses, Pb can cause retarded skeletal development. In mice, Pb can cause postimplantation mortality and skeletal malformations in fetuses. The common skeletal anomaly observed is the fusion of two or more cervical vertebrae (Jacquet and Gerber, 1979). Malformations in hamsters due to Pb exposure occur in the tail, ranging from stunting to complete absence of the tail (Ferm and Carpenter, 1967). In experimental animals, common malformations include brain defects, neural tube defects, and urogenital system and tail defects. It is important to mention that Pb has a greater potential for developmental neurotoxicity than for placental toxicity. For further details on placental, reproductive and developmental toxicity of Pb, refer to Flora *et al.* (2011).

## Mercury

Mercury (Hg) occurs naturally in the environment and exists in several forms. Common sources of Hg include mining, industrial, adhesives, fungicides and interior/exterior paints.

As a result, Hg is a widespread environmental contaminant that threatens the health of animals in general, and aquatic life and wildlife in particular. It is noteworthy that Hg is toxic in all forms. In most food-stuffs, Hg is found in the inorganic form. Fish, marine animals and some microorganisms have capability to convert elemental Hg to organic Hg, which accumulates in the food chain. In most of the poisoning incidents, MeHg was involved because of its popular use as a fungicide. During the outbreak of MeHg poisoning in Japan and Iraq, it was proven that there was transfer of MeHg through the placenta into the fetus. Following *in utero* exposure to MeHg, the target organ is the fetal brain. This is partly due to accumulation of Hg in the fetal brain because of its high requirement for protein synthesis. MeHg is readily absorbed and distributed throughout the body with various concentrations in

different tissues. In humans, brain MeHg levels can be as high as six-fold, compared with blood levels. This is in contrast to rats, which have a brain to blood ratio of 0.06, and mice with a ratio of 1.20. There is evidence that the placenta presents some barrier to Hg. By using the Gray PBPK model for MeHg, the placenta is modeled as four compartments with separate transfer constants for placental barrier and placental tissue transport. Generally, organic and metallic Hg crosses the placenta more readily than inorganic Hg. As a result, MeHg and metallic Hg accumulate in the fetus, whereas inorganic Hg concentrates in the placenta. Metallic Hg, after crossing the placenta, can be oxidized to  $\text{Hg}^{2+}$  in fetal tissues.  $\text{Hg}^{2+}$  accumulates in the placenta, where it inhibits the fetal uptake of certain essential metabolites or analogs of these metabolites.

MeHg has been found to produce embryotoxicity, fetotoxicity and teratogenicity in rats, mice, cats, guinea pigs and hamsters. In general, resorptions, dead fetuses and cleft palate are the most common findings. Some other common developmental effects include generalized edema, brain lesions, wavy ribs, asymmetric sternbrae and decreased ossification of parietal and occipital bones (Domingo, 1994). In essence, prenatal exposure to sufficient amounts of Hg results in developmental toxicity. In addition, Hg has a strong potential for neurotoxicity and neurobehavioral toxicity. For further details on placental, reproductive and developmental toxicity of Hg, refer to Ni *et al.* (2011) and the chapter on mercury in this book.

## PLACENTAL TOXICITY OF INSECTICIDES

Depending upon the duration, frequency and level of exposure, the insecticides of various classes (organophosphates, carbamates, organochlorines and pyrethroids) can adversely affect one or all three components of the maternal/placental/fetal unit. Numerous studies demonstrate that the insecticide residue is present in the exposed mother, placenta, cord blood, embryo and fetus, suggesting that the placenta and fetus are potentially exposed to these compounds. In this chapter, organophosphates (OPs) and carbamates (CMs) are discussed together because their mechanism of action and effects are quite similar (Gupta, 1995; Pelkonen *et al.*, 2006; Gupta, 2009; Gupta, 2011a; Gupta *et al.*, 2011).

### Organophosphates and carbamates

In general, both OPs and CMs exert their overt toxicity by inhibiting the activity of acetylcholinesterase (AChE).

Inactivation of AChE occurs owing to phosphorylation by OPs and carbamylation by CMs. Inhibition of AChE results in accumulation of acetylcholine (ACh), which overstimulates the muscarinic and nicotinic ACh receptors. There is evidence from a rat study that placenta may be a target of direct toxic effect by organophosphate pesticides (Levario-Carrillo *et al.*, 2004). By having active metabolic activity, the placenta can modulate the potency of OPs and CMs. The placenta can probably convert certain OPs of the "thioate" or "dithioate" group to their "oxon" analogs, which are many times more potent AChE inhibitors and thus more toxic than their parent compounds. In such circumstances, the placenta can be one of the several determining factors for fetal toxicity.

It is well established that an active cholinergic system exists in the placenta of some species and not in others, and there is evidence that a cholinergic system may have more than one function in placenta. In the placenta, ACh-like activity is highest in membranes, medium in the cotyledons and minimal in the cord, and it varies in the placenta as a function of gestational stage. The concentration of ACh in the placenta (a non-innervated tissue) is found to be greater than in various regions of the brain, and a marked decline occurs soon after parturition and expulsion of the placenta. The existence of the cholinergic system (using choline acetyltransferase activity, ChAT) has been studied in the term placenta of several species (monkey, mongoose, lemur, horse, cow, sheep, goat, pig, hamster, cat, rabbit, guinea pig and rat) (Hebb and Ratkovic, 1962). Literature abounds showing that the two species in which the placental cholinergic system has definitely been found are the two higher primates, man and monkey. In general, ACh has many functions in the placenta: (1) it acts as a local hormone related to uterine contractions and mechanisms associated with birth; (2) it controls permeability and transport; (3) it regulates blood flow and fluid volume in placental vessels; (4) it is involved in the mechanism of parturition; and (5) it acts as a local messenger molecule. ACh also seems to play a vital role in the maturation of the placenta. In addition, ACh may regulate trophoblastic channels, fluid balance and osmotic pressure, and therefore may influence contractile properties of myofibroblasts in the placenta. For further details on the roles of ACh, refer to Sastry (2000).

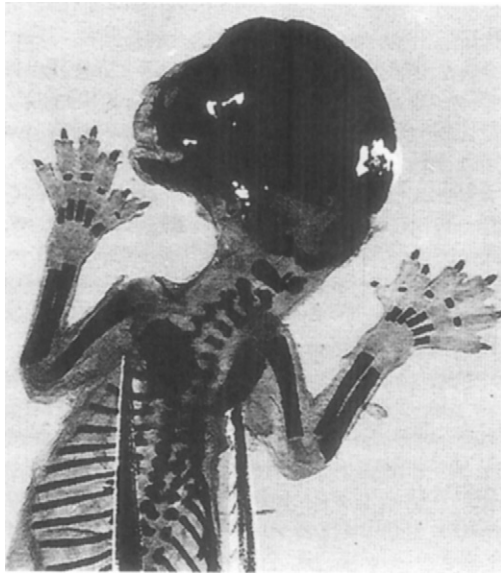
The placenta and fetus, by having AChE and other cholinergic elements (muscarinic and nicotinic ACh receptors), remain susceptible to OPs and CMs. Inhibition of AChE and BuChE activities can be used as a marker of exposure to an OP or CM, while AChE can also be used as a marker of effects. OPs and CMs readily cross the placental barrier and can act on the cholinergic and non-cholinergic components of the developing nervous system and other vital organs (Gupta *et al.*, 1984,

1985; Pelkonen *et al.*, 2006; Gupta, 2009). Significant inhibition of AChE and/or BuChE activities in maternal, placental and fetal tissues of rats and mice following prenatal exposure to several OPs (quinalphos, dicrotophos, methyl parathion, diazinon, etc.) has been demonstrated (Bus and Gibson, 1974; Srivastava *et al.*, 1992; Gupta, 1995; Abu-Qare and Abou-Donia, 2001; Gupta, 2009; Gupta *et al.*, 2011). Similar findings have been reported for CMs, including aldicarb, carbaryl, carbofuran and pirimicarb (Declume and Derache, 1977; Cambon *et al.*, 1979, 1980). From all these studies, AChE inhibition appears to be the major mechanism of toxicity. With either type of insecticides, the developing organism is much more sensitive to the induction of functional neural deficits. Furthermore, subchronic prenatal exposure to methyl parathion in rats resulted in altered postnatal development of brain AChE and ChAT activities, in addition to selected subtle alterations in behavior (Gupta *et al.*, 1985).

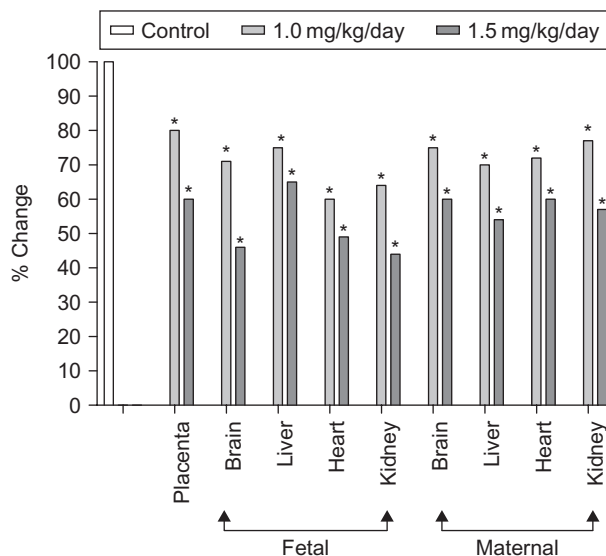
Anticholinesterase compounds have strong potential for embryotoxicity, embryoletality, fetotoxicity and teratogenesis. These effects vary depending upon the particular OP and CM involved. With these compounds, embryoletality is encountered so often that the expression of teratogenesis is rarely seen. In some experimental studies conducted in rats, mice, rabbits and hamsters, OPs at maternotoxic doses have been shown to produce developmental alterations, including growth retardation and embryotoxicity. Khera (1979) discovered polydactyly in fetuses of cats treated with dimethoate (12 mg/kg/day) during the 14th to 22nd day of pregnancy (Figure 20.1).

Methamidophos administration at a no-maternal toxicity dose (1 mg/kg, po) to female rats during GD 6–15 produced neither lethal effect on embryos nor caused congenital malformations at term; however, the embryo/fetal maturation process was significantly altered (De Castro *et al.*, 2000).

Wide species variability exists in sensitivity to OP-induced placental toxicity. It needs to be mentioned that the mechanisms involved in embryonic/fetal developmental malformation appear to be different from those involved in general toxicity. Alkylation of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) coenzymes by OPs is the major mechanism involved in the induction of teratogenesis (Schoental, 1977). In recent studies, DFP and carbofuran have been shown to cause marked depletion of NAD<sup>+</sup> in rats (Gupta *et al.*, 2001a, b). OPs are also known to cause alterations in the levels of RNA, glycogen, sulfated mucopolysaccharides and calcium in the developing tibiotarsus bones. Prenatal exposure to OPs has been shown to affect protein synthesis both *in vivo* (Gupta *et al.*, 1984) and *in vitro* (Welsch and Dettbarn, 1971; Marinovich *et al.*, 1996). The inhibitory effect on *in vivo* protein synthesis of methyl parathion (daily oral exposure throughout the period of organogenesis) was



**FIGURE 20.1** Fetal cat showing heptadactyly (right forepaw) and hexadactyly (left forepaw), from a cat exposed to dimethoate (Khera, 1979).



**FIGURE 20.2** Methyl parathion-induced inhibition of *in vivo* protein synthesis in placental, fetal and maternal tissues. An asterisk indicates a significant difference between methyl parathion-treated and control rats.

shown to be dose dependent, greater on day 19 than day 15 of gestation and more pronounced in fetal than in placental or maternal tissues of rats (Gupta *et al.*, 1984). The inhibitory effect on protein synthesis was more pronounced at a dose causing overt maternal toxicity (Figure 20.2). A mixture of OPs, including diazinon, dimethoate and azinphos, has been found to be more toxic to protein synthesis than any of the single OP compound alone (Marinovitch *et al.*, 1996).

Many placental toxicity studies conducted in mice, rats, hamsters and rabbits revealed no teratogenic responses to OPs. Among CMs, carbaryl is the only insecticide that has been studied in detail for placental toxicity. Like OPs, CMs have a greater potential for embryoletality and fetotoxicity and that precludes an expression of teratogenicity. Beagle dogs receiving carbaryl gave birth to fetuses with terata in 21 of a total of 181 pups. Fetal abnormalities included abdominal/thoracic fissures with varying degrees of intestinal agenesis and displacement, brachygnathia, ecaudate pups, failure of skeletal formation and superfluous phalanges (Smalley *et al.*, 1968). Carbaryl exposure during organogenesis produces terata in guinea pigs, but not in hamsters and rabbits. Other carbamates, such as carbofuran and propoxur, have not been found to be teratogenic. For further details on placental, reproductive and developmental toxicity of OPs and CMs refer to Gupta *et al.* (2011).

## Organochlorines

Organochlorine insecticides are classified into three groups: (1) dichlorodiphenylethanes (dichlorodiphenyl-trichloroethane, dicofol, methoxychlor and perthane); (2) hexachlorocyclohexanes (benzene hexachloride, chlordane, lindane, mirex and toxaphene); and (3) chlorinated cyclodienes (aldrin, dieldrin, endrin, chlordane, endosulfan and heptachlor).

The mechanism of action of organochlorines is not yet fully explained. The dichlorodiphenylethane (DDT type) compounds alter the transport of sodium and potassium ions across axonal membranes, resulting in an increased negative after-potential and followed by prolonged action potentials. As a result, repetitive firing and a spontaneous train of action potentials occur. Specifically, DDT inhibits the activation of sodium channels and the activation of potassium conductance. The mechanism involved in cyclodiene-induced hyperactivity of the CNS and convulsions can be explained based on their structural resemblance to the cyclic  $\gamma$ -aminobutyric acid (GABA) receptor antagonist picrotoxin. The mammalian GABA receptor is coupled to an intrinsic chloride ion channel and is the primary mediator of neuronal inhibition in the brain. Like picrotoxin, cyclodienes block the inhibitory action of GABA. Similar to other organochlorines, mirex and kepone cause stimulation of the CNS, hepatotoxicity and induction of the mixed-function oxidase system. In the CNS, symptoms observed in animals by cyclodiene organochlorines include tremors, convulsions, ataxia and changes in EEG patterns. The CNS symptoms could be due either to (1) inhibition of the  $\text{Na}^+/\text{K}^+$ -ATPase or the  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase activity, which can then interfere with nerve action or release of neurotransmitters, and/or (2) inhibition of the GABA receptor function.



The acute toxic signs associated with DDT and chlorinated benzene types of insecticides include paresthesia of the tongue, lips and face, apprehension, tremors and convulsions. Stimulation of the CNS is the most prominent effect. The acute signs and symptoms produced by cyclodienes include dizziness, nausea, vomiting, myoclonic jerking, motor hyperexcitability and seizures. Usually, epoxide metabolites of cyclodiene type of insecticides are much more toxic than their parent compounds. It is important to mention that the developing nervous system is the most sensitive target of organochlorine toxicity.

Only a few placental toxicity studies have been conducted with organochlorine compounds. In experimental studies, organochlorines, including DDT, DDE, DDD, dieldrin, endosulfan, methoxychlor and toxaphene, have been associated with estrogen-like effects in the reproductive system of laboratory animals. Exposure of female rats to methoxychlor before and during pregnancy can cause blockade of implantation, suppression of uterine decidualization, lack of corpora lutea and atresia of ovarian follicles. Exposure during preimplantation blocks implantation, whereas exposure during postimplantation causes fetal resorptions. Interference in the requisite hormonal milieu seems to be the major effect. Other effects of methoxychlor include estrogenic effect on uterine preimplantation differentiation, ovum transport rate, luteal regression and postimplantation decidual growth. In an experimental study, exposure of Swiss mice to lindane at different stages of pregnancy produced various toxicological effects, such as fetotoxicity and reproductive failure (Sircar and Lahiri, 1989). Lindane exposure during early pregnancy (days 1–4) caused total absence of any implantation; during mid-pregnancy (days 6–12) caused total resorptions of fetuses; and during late pregnancy (days 14–19) caused the death of all pups within 12 h to 5 days after parturition. Lindane can also cause reproductive failure by inducing a deficiency of steroid hormones (estrogen and progesterone).

Some studies in animals suggest that young animals exposed during gestation and infancy may be very sensitive to heptachlor and heptachlor epoxide. Changes in nervous system and immune function were found in these animals. Exposure to higher doses of heptachlor in animals can also result in decreases in body weight and death in animal newborn babies. A decrease in fertility and an increase in resorptions were observed in female rats acutely exposed to 1.8 mg/kg/day. Reduced fertility has also been observed in mice exposed to 8.4 mg/kg/day. A number of animal studies have demonstrated that exposure to heptachlor can result in decreased fertility and pregnancy losses. Impaired fertility was reported in female rats administered via gavage of 0.6 mg/kg/day heptachlor in groundnut oil for 14 days prior to mating (Amita Rani and Krishnakumari, 1995). In essence, though the exact mechanism in the

placental toxicity is yet to be elucidated, impaired fertility and pregnancy losses, in addition to the impaired development of the nervous system and immune system, have been found with organochlorines.

There are reports that dieldrin produced teratogenic effects, such as supernumerary ribs, with concomitant decrease in ossification centers in fetal hamsters (Chernoff *et al.*, 1975, 1979). In rats, exposure to mirex during pregnancy has been associated with perinatal deaths due to persistent cardiovascular problems, such as first- to third-degree fetal heart blockade (Grabowski, 1983). In addition, mirex causes altered lens growth and cataracts, along with other biochemical, physiological and histological changes (Rogers and Grabowski, 1983). Further details on reproductive and developmental toxicity of organochlorines can be found in a recent publication elsewhere (Malik *et al.*, 2011).

## Pyrethrins and pyrethroids

In recent years, the use of synthetic pyrethroids over other classes of insecticides has increased tremendously because of their selectively high toxicity to insects and low toxicity to mammals. Still, the risks to animal health exist because the products containing pyrethrins and pyrethroids are commonly used as ectoparasiticides. Exceeding the recommended levels of these insecticides or time of exposure often results in poisoning and deaths, especially in cats and dogs. Pyrethroids are of two types. Type I pyrethroids are those that lack  $\alpha$ -cyano moiety and give rise to the tremor syndrome (T syndrome). The syndrome includes the signs of whole body tremors, incoordination, prostration, tonic/clonic convulsions and death. A few common examples of type I are pyrethrin I, allethrin, tetramethrin, resmethrin and permethrin. Type II pyrethroids are those which contain  $\alpha$ -cyano moiety and cause the choreoathetosis/salivation (CS) syndrome. The CS syndrome is characterized by hyperactive behavior, profuse salivation, tremors, motor incoordination and hunch-backed posture. A few common examples of type II pyrethroids include cyphenothrin, cypermethrin, deltamethrin and fenvalerate.

Intoxication by pyrethroids results primarily from hyperexcitation of the nervous system. Type II syndrome involves primarily an action in the CNS, whereas with type I syndrome, peripheral nerves are also involved. Hyperexcitation of the nervous system is caused by repetitive firing and depolarization in nerve axons and synapses. Pyrethroids act directly through interaction with the sodium channel gating mechanism, thereby interfering with the generation and conduction of nerve impulses and inducing marked repetitive activity in various parts of the brain. Type I pyrethroids affect sodium channels in nerve membranes, causing repetitive

neuronal discharge and a prolonged negative after-potential, the effect being similar to those produced by DDT-type insecticides. Type II pyrethroids produce an even longer delay in sodium channel inactivation, leading to a persistent depolarization of the nerve membrane without repetitive discharge, a reduction in the amplitude of the action potential and eventually failure of axonal conduction and a blockade of impulses.

Prenatal or early postnatal exposure to pyrethroids (cypermethrin, fenvalerate and others) has been linked with significant neurochemical alterations in neonatal rats (Husain *et al.*, 1991; Malaviya *et al.*, 1993). Delayed maturation of the cerebral cortex occurs due to alterations in key enzymes of the neurotransmission process (e.g., monoamine oxidase, acetylcholinesterase and Na<sup>+</sup>/K<sup>+</sup>-ATPase). Prenatal exposure to these insecticides significantly delays differential responses in the levels of brain regional polyamines and ontogeny of sensory and motor reflexes in offspring. Other biochemical and neurochemical effects of these insecticides include impairment at the neurotransmitter receptors, including dopaminergic, cholinergic and catecholaminergic.

Compared to other classes of insecticides, pyrethroids are not well studied for placental toxicity because they are relatively less toxic to mammalian species. For example, permethrin at concentrations of 2000–4000 ppm showed only a week to moderate influence on *in utero* fetal development. Zhang *et al.* (2008) revealed that *cis* isomer of permethrin induced reproductive toxicity and not *trans* isomer. Female rats dermally exposed to cyhalothrin throughout the gestation period had offspring with delayed fur development, delayed ear and eye opening and delayed descent of the testes, but with no change in the age of vaginal opening. In adulthood, however, the sexual behavior of both male and female rats exposed to cyhalothrin prenatally is no different from that of control animals (Gomes *et al.*, 1991a, b). Prenatal exposure of rats to deltamethrin caused increase in early embryonic deaths and fetuses with retarded growth, hyperplasia of the lungs, dilatation of the renal pelvis and increase in placental weight (Abdel-Khalik *et al.*, 1993).

## Mycotoxins

Mycotoxins are secondary metabolites of fungi and several of them are found to be toxic *in utero* in both man and animals. In general, mycotoxins can produce placental toxicity by one or more of three common mechanisms. In brief, mycotoxins can interfere with hormonal activity, which can damage parental gametes, producing infertility or abnormal offspring if successful fertilization occurs. Mycotoxins can cause fetal malformations, especially during organogenesis, by interfering with fetal nucleic acid and protein biosynthesis. This interrupts normal

cell differentiation and organogenesis, resulting in fetal malformations. Mycotoxins can also affect the fetus indirectly by affecting vital maternal organs, such as the liver, reducing nutrient transfer to the fetus or increasing transfer of toxicants. Some of the common mycotoxins that cause placental toxicity are briefly discussed below.

## Aflatoxins

Aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>) are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which commonly contaminate a variety of animal food. Aflatoxins are proven to be mutagens, carcinogens and teratogens. Some of the aflatoxins and their metabolites are known to cross the placental barrier in humans as well as in animals. From limited studies conducted on laboratory animals, aflatoxin B<sub>1</sub> and G<sub>1</sub> appear to have strong potential for embryocidal, fetotoxic and teratogenic effects. Aflatoxin B<sub>1</sub> and G<sub>1</sub>, with varying doses administered orally or ip in mice on gestation day (GD) 12 and 13, produced moderate retardation in fetal development, cleft palate and changes in diaphragm. Aflatoxin G<sub>1</sub> also produced malformations in the kidneys (Roll *et al.*, 1990). Pregnant rats exposed to aflatoxin B<sub>1</sub> via the oral route have been shown to result in significant anti-implantational activity and loss of viability among the litters (Choudhary *et al.*, 1992). For the reproductive and developmental effects of aflatoxins refer to a recent publication elsewhere (Gupta, 2011b).

## Fumonisin

Fumonisin (FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub>, FB<sub>4</sub>, FA<sub>1</sub>, FA<sub>2</sub>, C<sub>1</sub> and several others) are produced by *Fusarium* fungi, primarily *F. verticillioides* (formerly *F. moniliforme* Sheldon) and *F. proliferatum*. These mycotoxins are found in corn and therefore contaminate corn-based food/feed worldwide. Over 28 fumonisins have been identified, but FB<sub>1</sub> and FB<sub>2</sub> are the most abundant and most toxic mycotoxins of this group. Fumonisin-induced toxic effects appear to be species specific. Consumption of fumonisin-contaminated food/feed has been associated with many organ-specific diseases. For example, esophageal cancer in man in the Transkei region in South Africa and Linxian County in China, hepatopathy and hepatocarcinoma in laboratory rats, porcine pulmonary edema (PPE) in swine, and equine leukoencephalomalacia (ELEM) or moldy corn poisoning in equidae. In addition, fumonisins produce mild to fatal toxicity in the liver, kidney and heart of rats and horses. Among all species, equidae is the most sensitive because fumonisins affect the brain of this species.

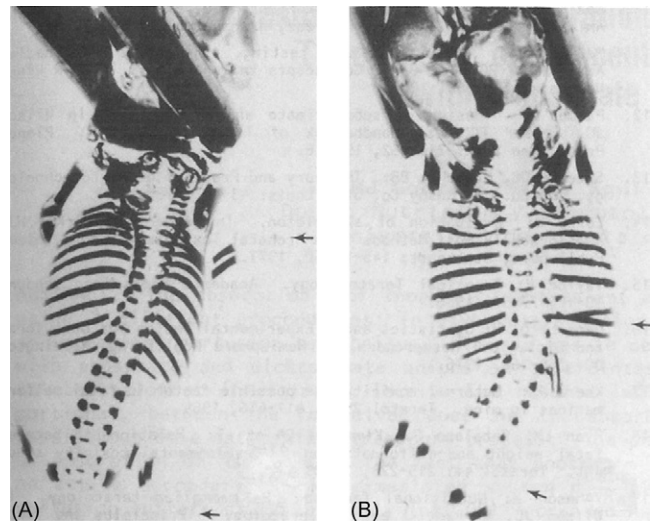
Experimental animal studies and field-case findings revealed that fumonisins have a strong potential for

developmental toxicity and teratogenicity (Voss *et al.*, 2011). Purified FB<sub>1</sub> and *F. proliferatum* culture extract have been shown to be embryotoxic when inoculated into incubated eggs (Javed *et al.*, 1993a, b). In addition to mortality, which was dependent on the dose and time of administration (i.e., day 1 or 10), embryonic changes occurred, including hydrocephalus, enlarged beaks and elongated necks. In hamsters, FB<sub>1</sub> caused increased incidence of prenatal losses, including deaths and resorptions. At a dose of 12 mg/kg, all litters were affected and all the fetuses were dead and resorbed, without any clinicopathological evidence of maternal toxicity. Fetal resorptions were associated with a greater severity of placental necrosis. Placental tissues showed signs of degeneration and involution, focal distortion and necrosis of the trophoblastic layer, especially at the periphery, and hemorrhage and necrosis at the base of the placenta. Placentas had extensive necrosis and fetal capillaries in the hemochorial portion, with concomitant collapse of the stroma. Such changes were consistent with the embryo-lethal effects of FB<sub>1</sub>. External malformations include hooked or curled tail, ectrodactyly of the front/rear limbs and cleft palate. Developmental effects of aqueous culture extract of *F. moniliforme* in CD1 mice are reported to be due to FB<sub>1</sub>. The effects include maternal deaths, reduced maternal body weight gain, increased embryonic resorptions, reduced pup weights and fetal malformations, such as cleft palate, hydrocephalus and ossification deficits (Gross *et al.*, 1994; Reddy *et al.*, 1995).

FB<sub>1</sub> is found to be fetotoxic in F344/N rats. Pregnant rats receiving FB<sub>1</sub> at a dose of 30 or 60 mg/kg, po (GD 8–12) developed significant impairment of ossification of the sternebrae and vertebral bodies in their fetuses (Lebepe-Mazur *et al.*, 1995). It appears that FB<sub>1</sub> produces fetotoxicity in rats by suppressing growth and fetal bone development (Figure 20.3). However, in Sprague-Dawley rats, there is little or no evidence of embryotoxicity or fetotoxicity by FB<sub>1</sub>, especially in the absence of maternal toxicity (Ferguson *et al.*, 1997). Pregnant New Zealand white rabbits receiving FB<sub>1</sub> showed no evidence of embryotoxicity or teratogenicity, despite the fact that the pregnant rabbits were very sensitive to FB<sub>1</sub> (LaBorde *et al.*, 1997).

In pregnant mares, late term abortions have been observed by consuming fumonisin- contaminated corn/corn-based feed. The surviving colts are usually slow learners and behaviorally abnormal. Suppressed growth, bone development and immune system appeared to be the major contributing factors. Abortions have also been observed in pregnant sows by consuming fumonisin-contaminated corn that caused hypoxia resulting from maternal respiratory distress (Harrison *et al.*, 1990).

Although the exact mechanism of action involved in fumonisin toxicity is yet to be established, it has been demonstrated that fumonisins inhibit sphinganine/sphingosine



**FIGURE 20.3** Skeletal development of control- (A) and fumonisin- (B) treated rat pup. A = pup from a control dam, with normally shaped ribs and normal ossification of the phalanges and vertebrae bodies. B = pup from a dam treated with 60 mg/kg fumonisin B<sub>1</sub>, having under-ossification of the vertebral bodies of the cervical and lumbar regions as well as the phalanges (Lebepe-Mazur *et al.*, 1995).

N-acetyl transferase (ceramide synthase) activity, and consequently increase the concentrations of sphinganine to sphingosine in target organs of many species (Wang *et al.*, 1992; Riley *et al.*, 1993). Accumulation of sphinganine may be responsible for cell death because long-chain bases such as sphinganine and sphingosine are known protein kinase C inhibitors and can be cytotoxic. It is suggested that an abnormal sphinganine/sphingosine ratio in the fetus, while normal ratio in the dam, may attest to the unique sensitivity of the conceptus to fumonisins. For further details on placental, reproductive and developmental toxicity of fumonisins, refer to Voss *et al.* (2011) and the chapter on fumonisins in this book.

## Ochratoxin A

Ochratoxin A (OTA) is produced by several *Aspergillus* and *Penicillium* species. This mycotoxin is known to contaminate food/feed and thereby cause a variety of adverse health effects (nephrotoxicity, carcinogenicity, teratogenicity and immunotoxicity), nephrocarcinogenicity being the most prominent. OTA has been demonstrated to cause carcinogenesis, immunotoxicity and teratogenesis in rats and rabbits (Mayura *et al.*, 1982a; Wangikar *et al.*, 2004). In general, the underlying mechanisms involved in the toxicity of OTA include: (1) inhibition of protein synthesis, (2) increased lipid peroxidation and (3) partial inhibition of ATP-dependent calcium uptake and inhibition of the cell-mediated immune



response. In addition, OTA inhibits the phosphorylase enzyme system, possibly by competing with 3',5'-cyclic AMP for phosphorylase b kinase activity. In the kidney, OTA specifically affects organic anion transport, but not organic cation transport, in both the brush border and basolateral membranes, which explains its entry into the renal cells and the development of toxicity. Gene expression study indicates that OTA-induced renal toxicity involves the impairment of defense potential causing oxidative damage that eventually leads to cell proliferation and cancer. Epigenetic mechanism appears to be highly probable (Marin-Kuan *et al.*, 2006)

A recent toxicokinetic study in rats revealed that bioavailability of OTA is significantly greater in adult males than in females and young animals (Vettorazzi *et al.*, 2010). Fasting further enhances the oral absorption of OTA. Following absorption, OTA transplacentally traverses in rodents and other species and causes fetal death in laboratory and domestic animals (Still *et al.*, 1971). Pregnant rats receiving a culture of *A. ochraceus* on GD 10 showed embryo resorptions and fetal deaths. Subsequent studies in rats further demonstrated that a high dose of purified OTA administered on GD 10 also caused a large number of fetal deaths and embryo resorptions. However, in sheep, placental transfer of OTA was found to be in trace amounts resulting in very little or no evidence of fetal toxicity or teratogenesis. Rabbits receiving a combination of OTA and aflatoxin B<sub>1</sub> showed antagonistic interaction, yet revealed a characteristic cardiac anomaly with a valvular defect at the auriculo-ventricular junction (Wangikar *et al.*, 2005). For further details of OTA toxicity in general and reproductive and developmental toxicity in particular, refer to Gupta (2011b) and the chapter on OTA in this book.

## Rubratoxins

Both rubratoxin A and B are produced by *Penicillium rubrum* and *P. perpurogenum*. Rubratoxin B is the primary mycotoxin that causes hepatotoxicity, nephrotoxicity and splenotoxicity in several animal species. In both laboratory and domestic animals, typical signs of poisoning are hepatic failure, coagulopathy, hemorrhage of the gut mucosa, bloody feces and death. Rubratoxin B<sub>1</sub> has been demonstrated to be a potent mutagen and teratogen in laboratory animals (Wilson and Harbison, 1973). Although the underlying mechanism in rubratoxin-induced placental toxicity is yet to be explained, rubratoxin B is known to produce gross malformations, internal anomalies, skeletal malformations, embryo lethality, intrauterine growth retardation, increased skeletal variations and delayed ossification in the mouse fetuses. The teratogenic effects in near-term fetuses include exophthalmos, missing ears, spina bifida,

microphthalmia, anophthalmia, short tail, exencephaly and hydramnion.

## Secalonic acid D

Secalonic acid D (SAD) is produced by *Penicillium oxalicum*. In rats and mice, SAD is known to cause embryotoxicity, embryoletality, fetotoxicity and teratogenicity. Pregnant CD1 mice receiving SAD (>5mg/kg/day, ip, GD 7–15) showed a reduction in maternal body weight gain, and an increase in resorptions of implanted embryos (Reddy *et al.*, 1981). The resorptions rate was found to be 100% at 15mg/kg dose. Multiple gross, skeletal and visceral anomalies were noted in fetuses born to mothers receiving 10mg/kg or more. Major malformations included cleft palate, cleft lip, open eyelids, missing phalangeal ossification centers and shortened mandibles. In CD1 mice, SAD is proved to be an embryocidal, fetotoxic and teratogenic. Pregnant rats receiving SAD as a single sc dose (25mg/kg) on one of GD 6–10, 12 or 14, or 15mg/kg on GD 10, produced fetotoxic and teratogenic effects, although the effects were less marked with the lower dose (Mayura *et al.*, 1982b). The highest number of resorptions, greatest depression of fetal body weight and largest number of malformations occurred when SAD was injected on GD 10. The major gross malformations were anophthalmia (GD 9 and 10), exencephaly (GD 9) and defects in limbs, digits and tail (GD 10). The major skeletal defects involved the vertebrae and ribs. The major internal soft tissue defects were hydronephrosis (GD 9 and 10), tracheo-esophageal fistula and renal agenesis (GD 10). In essence, pregnant rats are most sensitive to SAD exposure on GD 10 for fetotoxicity and teratogenicity.

## Trichothecenes

The trichothecenes are a group of structurally related mycotoxins with varying degrees of cytotoxic potency. Diacetoxyscirpenol (DAS), T-2 toxin and deoxynivalenol (DON or vomitoxin) are the three major mycotoxins of this group that are commonly encountered in animal poisonings. T-2 and DAS are produced by *F. sporotrichioides* and vomitoxin is produced by *F. roseum*. There is strong evidence that trichothecenes cross the placental barrier and can cause both embryonic death and structural malformations when administered to laboratory animals during pregnancy (Francis, 1989). Since domestic and avian species are exposed to trichothecenes through consumption of contaminated feed, and trichothecenes in general cause feed refusal, it is expected that the induced maternal/fetal toxicity be minimal. Furthermore, *in vivo* studies with different animal



species indicate that orally or parenterally administered trichothecenes do not bioaccumulate due to their short elimination half-lives: <30min for T-2 toxin in swine, cattle and dogs (Yagen and Bialer, 1993), and 3–5 h for DON in swine and cattle (Rotter *et al.*, 1996).

## DON

Feeding of a deoxynivalenol (DON, vomitoxin)-contaminated diet at 5ppm to rats throughout gestation does not adversely affect pregnancy or increase birth defects (Morrissey, 1984). Vomitoxin does not produce teratogenesis even at a 20ppm level. However, at this dose it can decrease the rate of pregnancy. Mice intubated with vomitoxin (2.5 and 5mg/kg) on GD 8–11 produced embryoletality, and at doses of 5mg/kg or greater produced embryoletality (Khera *et al.*, 1982). In mice, vomitoxin at a dose of 10mg/kg caused maternal mortality. Fetal mortality by vomitoxin is dose dependent (100% at 10mg/kg, 80% at 5mg/kg and 72% at 2.5mg/kg). However, none of the doses of vomitoxin induced damage to the placenta or teratogenesis in mice.

## DAS

There is very little known about placental toxicity of diacetoxyscirpenol (DAS). In rats, DAS at a dose rate of 2, 3 or 6mg/kg ip given on one of GD 7–11 caused no maternal toxicity (Mayura *et al.*, 1985). The highest dose of DAS caused 100% resorptions, while with the lowest dose, the surviving pups showed a variety of malformations, including hydrocephaly and exencephaly.

## T-2 toxin

T-2 toxin is the most potent mycotoxin of the trichothecenes group, and has been involved in mass poisoning in animals (Hsu *et al.*, 1972). It has been well established that T-2 toxin crosses the placenta (Hayes, 1981). In rats, T-2 toxin produced behavioral teratogenesis, when the toxin was given prenatally (Francis, 1989). Mice exposed to a single dose of T-2 toxin (3mg/kg) on GD 7, 8, 10, 11 or 12, showed maternal toxicity with 17% mortality and whole litter resorptions (Roussoux *et al.*, 1985). Maternal death was typically due to placental hemorrhage, and surviving pups showed retardation defects of the skeletal system, exencephaly, abdominal defects and cleft palate. The findings of a comprehensive study in mice treated with T-2 toxin (0.5, 1.0 or 1.5mg/kg, ip) on GD 7, 9, 10 or 11 are briefly described in Stanford *et al.* (1975). Treatment on GD 9 reduced the survival rate to 85% at 1mg/kg and to 8% at 1.5mg/kg, without any evidence of teratogenesis in surviving pups. Among the

surviving pups of dams treated at 1.0 or 1.5mg/kg on GD 10, 37% grossly malformed and 42% had skeletal malformations. Commonly observed malformations were exencephaly, congenitally open eyelids and retarded jaws. Treatment on GD 11 resulted in greater embryotoxicity (75% deaths at 1mg/kg and 100% deaths at 1.5mg/kg), but the malformation rate among surviving pups was not greater than 13% at any dosage. In another study, mice given T-2 toxin in feed at 5 and 10ppm throughout gestation resulted in whole litter abortions in 67 and 100% of the dams, respectively (Francis, 1989). Delayed ossifications of the coccyx and growth retardation were the prominent effects. Overall, the observed effects were cumulative and dose dependent.

It appears that the trichothecenes induce embryotoxicity at maternotoxic doses in laboratory animals. Death of the embryo/fetus is the most common finding. The surviving pups rarely show frank malformations of nervous system and skeletal system.

For further details on trichothecenes toxicity in general and reproductive and developmental toxicity in particular, refer to Mostrom (2011) and Chapter 94 in this book.

## Zearalenone

Zearalenone is produced by the fungi *Fusarium graminearum*, *F. culmorum* and other *Fusarium* species. It is important to study the placental toxicity of zearalenone and its two major metabolites ( $\alpha$ - and  $\beta$ -zearalenol) as they bind to estrogen receptor and produce a variety of reproductive problems. Zearalenone has been found to cross the placenta and reach fetuses of mice and rats (Appelgren *et al.*, 1982; Bernhoft *et al.*, 2001). In maternal tissues, zearalenone and its metabolites accumulate in the liver, but in fetal tissues it is yet to be established. Due to its estrogenic activity, zearalenone is known to perturb the ovulation cycle and reduce litter size in domestic animals, particularly swine. Similar findings have been noted in experimental studies conducted in swine, mice and rats, while exposure to zearalenone during pregnancy reduces fetal survival (Kuiper-Goodman *et al.*, 1987). The structurally related compound  $\alpha$ -zearalanol, which is used for growth promotion in cattle, has been shown to accelerate testicular development and exert alterations in Leydig cells in mice after *in utero* exposure (Perez-Martinez *et al.*, 1997).

## TOBACCO

Tobacco plants and their products contain many toxic alkaloids, but nicotine is believed to be the major component causing alterations in embryo and fetal development

that leads to teratogenesis. Pets are exposed to tobacco by ingesting commercial products (e.g., cigarettes, chewing tobacco, etc.), whereas livestock are exposed by consuming discarded tobacco stalks or contaminated hay with tobacco plant dripping in the barn. Most often, poisoning encountered in animals with tobacco is acute in nature, exhibiting the signs of neurologic and muscular disturbances.

The pharmacological and toxicological effects of nicotine are dose dependent and primarily occur in the CNS, cardiovascular system, skeletal muscles and GI tract. Nicotine is a rapidly acting sympathetic and parasympathetic ganglionic depolarizer. In small doses, nicotine stimulates the autonomic ganglia, while in larger doses it blocks the ganglia. Similarly, in skeletal muscles small doses of nicotine initially stimulate the nicotinic receptors of the motor end plate, while larger doses block these receptors. Cardiovascular signs, such as tachycardia and hypertension, result from stimulation of sympathetic ganglia and the adrenal medulla. Death occurs from paralysis of respiratory muscles and cardiac arrest.

Some tobacco alkaloids, especially nicotine, have been studied for their distribution, metabolism and elimination in animals. It is also established that some of these alkaloids cross the placenta and produce teratogenic effects (Suzuki *et al.*, 1974; Sastry and Janson, 1995; Panter *et al.*, 1999; Czekaj *et al.*, 2002). In some studies, tobacco components have been shown to produce deleterious effects on embryonic development in the early stages of pregnancy, before the placenta is fully formed. In mice, nicotine increases  $[Ca^{2+}]_i$  and reactive oxygen species levels, which play a role in nicotine-induced embryonic apoptosis and malformations, and that eventually leads to teratogenesis (Zhao and Reece, 2005). Swine appears to be the most sensitive species in the context of teratogenesis. The fetal arthrogryposis occurs without signs of intoxication in the dams and without fetal deaths or abortions, but the deformed piglets usually die shortly after birth. With *N. glauca* fetal deformities are preceded by the appearance of signs of acute toxicity in the dam (Keeler *et al.*, 1981). Deformities in the newborn following *in utero* exposure include severe flexure and lateral rotation of the carpal joints, moderate rotation of the fetlocks and less commonly spinal malformations resulting in lordosis or scoliosis, and cleft palate (Burrows and Tyrl, 2001). Exactly, which component(s) of the tobacco is involved in inducing teratogenesis is yet to be confirmed.

## CONCLUSIONS AND FUTURE DIRECTIONS

Until recently, placental toxicity has received very little attention compared to fetal or maternal toxicity. This is

due to the fact that placenta is discarded at the termination of pregnancy. However, healthy placenta with normal structure and function is required for proper supplies of nutrition to the fetus and its protection from adverse conditions. It is important to mention that the placenta is also a source of several growth factors that are required by the fetus. The placenta by having an active metabolism appears to influence fetal toxicity of chemicals. Many chemicals cross the placenta and produce deleterious effects on the fetus, while other chemicals cause damage to the placenta and thereby adversely affect the fetus. Detailed studies need to be done to understand exactly how the toxicants from various classes interfere with the structure, function and development of the placenta. As much emphasis should be given to explore the molecular mechanism(s) involved in deleterious effects of chemicals in the placenta as has been given to the fetus or mother.

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## Dermal toxicity

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### INTRODUCTION

Skin is an essential and dynamic organ. In addition to performing important functions including thermoregulation and preventing insensible water loss, it also has important metabolic, immunological and neurosensory properties. However, the predominant function of skin is to protect the body against a variety of toxicological insults. Animals are relatively less protective against such insults as compared to humans due to lack of clothing, inferior housing and different social interactions. In most instances, the skin of animals directly contacts environmental, chemical and other pollutant exposure without the benefit of man-made protection. Although the largest organ of the body can often face these insults to a certain threshold, animals exhibit symptoms of dermal toxicity when this limit is passed.

Knowledge of the basic structure of skin is necessary to understand the mechanisms of dermal absorption and toxicity of topically applied toxicants. Skin is composed of three distinct layers, namely, epidermis, dermis and hypodermis. Of these layers, the epidermis (consists of keratinocytes and non-keratinocytes) is considered the most important barrier to dermal penetration of most chemicals. The epidermal layers can further be classified from external surface inward as stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The stratum basale consists of a single layer of cuboidal or columnar cells resting on basal lamina and is the viable layer of cells in the epidermis. These cells are mitotically active, thus keeping the epidermis replenished as the stratum corneum (SC) cells are sloughed from the surface epidermis (Lavker and Sun,

1983; Monteiro-Riviere, 2006). The approximate cell turnover and self-replacement time in normal human skin is 28 days. This differs widely across species. Mechanical or chemical injuries can increase the mitotic rate of basal cells. Stratum spinosum consists of several layers of irregular polyhedral cells. The cells of this layer have numerous tonofilaments and small membrane bound organelles (lamellar granules). Stratum granulosum consists of several layers of flattened cells containing irregularly shaped, non-membrane bound and electron-dense keratohyalin granules. These granules have a role in keratinization and maintaining the barrier functions of the skin. The lamellar granules contain several types of lipids (ceramides, cholesterol, fatty acids) and hydrolytic enzymes including proteases, acid phosphatases, lipases and glycosidases (Downing, 1992; Monteiro-Riviere, 2006). Stratum lucidum is a thin layer only present in very thick areas of skin such as palmer and plantar surfaces.

The outermost layer of the epidermis is the SC which consists of several layers of completely keratinized dead cells (corneocytes) embedded in an extracellular lipid matrix. This has been depicted as the “bricks and mortar model” where keratinized cells, the bricks, are embedded in the lipid mortar. The dead keratinized cells are highly water absorbent and keep the skin moist and soft. The water-holding capacity of the epidermis is further maintained by sebum (natural oil covering the skin) secretion from glandular structures of the skin. A number of non-keratinocyte cell types are found in skin. The melanocytes are involved in skin pigmentation; Merkel cells act as mechanoreceptors for touch, while Langerhans cells play a major role in the skin immune response. These specialized cells are not involved in barrier functions of skin (Monteiro-Riviere, 2006).

Other specialized regions of the epidermis are the skin appendages which include hair, sweat and sebaceous glands, hoof, claw, nail, feathers and horn. The dermis consists of dense irregular connective tissue with collagen, elastic and reticular fibers in a mucopolysaccharides ground substance. Fibroblasts, mast cells and macrophages are the predominant cells of this layer. In addition, sweat glands, sebaceous glands, hair follicles and erector pili muscles are located in the dermis. The hypodermis is a layer of loose connective tissues beneath the dermis. This layer helps to anchor the dermis to the underlying tissues such as muscle or bone (Monteiro-Riviere, 2006).

SC is considered the primary barrier of skin against the penetration of foreign substances as this layer provides up to 1000 times the resistance to exogenous compounds as compared to the layers beneath it. Disruption of this layer either by physical (tape stripping) or by chemical means will adversely affect the barrier properties. Extraction of these epidermal lipids with organic solvents (Monteiro-Riviere *et al.*, 2001) and jet fuel hydrocarbons (Muhammad *et al.*, 2005b) can reduce the barrier functions of the skin. The other two deeper layers of skin (dermis and hypodermis) offer no resistance to penetration of most compounds. Once a substance has penetrated the epidermis, it will easily traverse the other layers. The potential exceptions are very lipophilic compounds (log lipid to water partition coefficient, greater than four) that may tend to stay in the lipid environment of skin.

## ABSORPTION

Toxicants traverse biological membranes either by passive diffusion or by active transport. A passive diffusion process implies that the solute flux is linearly dependent on the solute concentration gradient; while active transport processes typically involve a saturable mechanism (Friedman, 1986). Dermal absorption is a passive process. Generally, topical absorption involves a sequence of events that include partitioning of the molecule into the SC from the applied vehicle phase, diffusion through the SC, partitioning from the SC into the viable epidermis, diffusion through the epidermis and upper dermis and finally capillary uptake and systemic exposure. Hence, the movement of toxic molecules across the skin involves transport through a series of resistances. In general, for polar toxicants, the diffusional resistance of the SC is large compared to that presented by the viable epidermis and dermis. For more lipophilic molecules, the resistance of the SC is smaller. However, the SC maintains a rate-controlling role since a highly lipophilic molecule does not favorably partition out of the SC into the more aqueous viable epidermis. From the description of

SC components and structure, one can envisage that a diffusing molecule can adopt one or more of the following penetration pathways:

- 1 The intercellular/paracellular path, via the tortuous but continuous intercellular lipids.
- 2 The transcellular path, indicating that the toxicants transfer sequentially and repeatedly through the "bricks" and "mortar."
- 3 The transappendageal path via hair follicles, sweat pores, etc.

Most molecules follow the first penetration pathway, yet the absorption of certain compounds can take place via a transfollicular path or sweat pores, often resulting in skin penetration (residing within skin) rather than true absorption (systemic exposure). Both absorption and penetration are important determinants of direct chemical toxicity to the skin. Diffusion occurs through the intercellular lipids, hence partitioning into these lipids is often the primary determinant of absorption. Species differences in lipid makeup thus also translate into species differences in absorption.

Percutaneous absorption through the intercellular pathway of the SC is driven by passive diffusion down a concentration gradient described at steady state by Fick's law of diffusion (Riviere, 1999):

$$\text{Flux} = [(D \cdot PC \cdot SA) / H](\Delta x)$$

where  $D$  is the diffusion coefficient and  $PC$  is the partition coefficient,  $SA$  is the applied surface area,  $H$  is membrane thickness (or more precisely the intercellular path length) and  $\Delta x$  is the concentration gradient across the membrane. Since *in vivo* blood concentrations after absorption are negligible compared to applied surface concentration,  $\Delta x$  reduces to the concentration ( $C$ ). It is this relationship that allows the prediction of compound flux across the skin to be correlated to factors predictive of  $D$  and  $PC$  (e.g., octanol/water partition coefficients reflecting chemical partitioning into the SC lipids). Flux is expressed in terms of applied surface area, often normalized to  $\text{cm}^2$ .

The term  $(D \cdot PC / H)$  is compound dependent and is termed the permeability coefficient ( $K_p$ ), reducing the determination of flux to  $K_p \cdot \Delta X$  or  $K_p \cdot C$ , a first-order pharmacokinetic equation ( $dx/dt = kX$ ). Rearrangement of this equation yields the primary method used to experimentally determine  $K_p$ :

$$K_p = \text{steady state flux / concentration}$$

It must be stressed that both transdermal flux and  $K_p$  are not only chemical dependent, but also tightly

constrained by the membrane system studied as well as the method of topical application (neat compound, vehicle, length of experiment, etc.). The PC that is integral to  $K_p$  is the PC between the surface or applied vehicle and the SC. Different vehicles will thus result in different PCs. Similarly, skin from different species may result in different PCs due to differences in the SC lipids and intercellular path lengths.

Cutaneous biotransformation of drugs is also a barrier to absorption of certain compounds, and has been used to promote the absorption of certain chemicals (pro drugs) across the skin. This aspect is important in skin toxicology as non-toxic parent compounds may be bioactivated within the epidermis, such as benzo[a]pyrene to an epoxide (Riviere, 1999).

There are numerous factors that can affect the dermal toxicity in animals. These include the species, breed, age, health status, skin condition (dryness, hairiness or thickness) and local environment (weather, humidity, temperature). Some of these factors including epidermal thickness, epidermal cell size, number of cell layers and blood flow patterns can vary between species as well as within the species (Monteiro-Riviere *et al.*, 1990). As a general rule, young and emaciated animals are more prone to dermal intoxication than are adults or healthy animals. In this chapter, the agents involved in dermal toxicity directly or indirectly are described. There are a variety of compounds including pesticides, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), heavy metals, photosensitizing agents and toxic plants that can affect the skin of animals by multiple mechanisms.

## PLANTS CAUSING DERMAL TOXICITY IN ANIMALS

Exposure to plant toxins may be the most common cause of dermal toxicity in animals, since the majority of production animals are reared on pastures where they come in contact with various types of vegetation. Some plants have compounds that directly affect the skin after contact, while others have thorns/spines that can cause mechanical injury to the skin. Many compounds may induce allergic dermatitis, similar to poison ivy in humans. A discussion of contact sensitization is beyond the scope of the present chapter. A text in immunology or allergy medicine should be consulted. Many plants have pigments or compounds that once absorbed from the gastrointestinal tract, induce a direct effect on non-pigmented skin when exposed to light. Other plants can result in a secondary photosensitization by virtue of toxic alkaloids that cause irreversible liver disease.

## PHOTOSENSITIZATION

Photosensitization is a severe dermatitis resulting from a complex reaction induced by plant pigments exposed to ultraviolet (UV) sunlight in the skin of animals that have eaten certain plants (Rowe, 1989). UV radiation can induce acute or chronic photobiologic reactions in the absence of exogenous chromophores such as nuclear DNA (Horio *et al.*, 2005). Non-pigmented skin gives the most severe reaction where these reactive compounds are directly exposed to UV light, most likely secondary to light-enhanced photooxidation. The amino acids susceptible to oxidation (histidine, tyrosine and tryptophan) once oxidized evoke an intense inflammatory response in the blood vessels and surrounding cells resulting in tissue necrosis. In addition to plant pigments, fungal toxins and certain chemicals can induce photosensitization. Quite frequently horses and cattle develop photosensitization during grazing on pasture which may be due to oxidative damage in photodynamic therapy (Clare, 1955; Babilas *et al.*, 2005). Similarly outbreaks of facial eczema (pithomycotoxicosis), a hepatogenous photosensitization caused by the mycotoxin sporidesmin, have been reported in ruminants in the Azores Islands of Portugal (Pinto *et al.*, 2005).

Photosensitization may be classified into primary and secondary types (Table 21.1). Primary photosensitization is associated with photodynamic compounds in certain plants, which react in the non-pigmented skin with UV light to cause a severe dermatitis. Secondary photosensitization results when an animal's liver is sufficiently diseased to be unable to remove plant byproducts that can react with UV light to cause photosensitization. Phylloerythrin, a bacterial breakdown product of chlorophyll, is a potent photosensitizing compound. Normally phylloerythrin is removed by the liver and is excreted in the bile, but if the liver is severely diseased, it accumulates in the blood to cause photosensitization if a white skinned animal is exposed to UV light. Secondary photosensitization is much more common in livestock than primary photosensitization.

Primary photosensitization develops when animals eat plants having polyphenolic pigments. Historically, two plants, buckwheat (Kingsbury, 1964) and St. John's wort (Marsh, 1930; Araya and Ford, 1981), are associated with primary photosensitization. Bishop's weed (*Ammi majus*), rain lily (*Cooperia pedunculata*), spring parsley (*Cymopterus watsonii*), buckwheat (*Fagopyrum esculentum*), giant hogweed (*Heracleum mantegazzianum*), St. John's wort (*Hypericum perforatum*) and Dutchman's britches (*Thamnosma texana*) are reported as primary photosensitizing plants (Knight and Walter, 2003). These plant species (Table 21.1) contain photodynamic furanocoumarin compounds that have been associated with



TABLE 21.1 Important plants causing photosensitization in animals

Common name	Botanical name	Type of photo-sensitivity
St. John's wort	<i>Hypericum perforatum</i>	Primary
Spring parsley	<i>Cymopterus watsonii</i>	Primary
Bishop's weed	<i>Ammi majus</i>	Primary
Dutchman's britches	<i>Thamnosma texana</i>	Primary
Rain lily	<i>Cooperia pedunculata</i>	Primary
Groundsels, Senecio	<i>Senecio</i> spp.	Secondary
Fiddle neck	<i>Amsinckia</i> spp.	Secondary
Rattle box	<i>Crotolaria</i> spp.	Secondary
Hound's tongue	<i>Cynoglossum officinale</i>	Secondary
Blue weed	<i>Echium vulgare</i>	Secondary
Lantana	<i>Lantana camara</i>	Secondary
Panic grasses	<i>Panicum</i> spp.	Secondary
Horse brush	<i>Tetradymia</i> spp.	Secondary
Alsike clover	<i>Trifolium hybridum</i>	Secondary
Black sage	<i>Artemisia nigra</i>	Secondary
Puncture vine	<i>Tribulus terrestris</i>	Secondary

photosensitivity through ingestion and direct contact with the skin (Dollahite *et al.*, 1978; Oertli *et al.*, 1983). A seasonal photosensitivity of cattle, associated with the consumption of the dead leaves of rain lily has been reported in southeast Texas (Rowe *et al.*, 1987; Casteel *et al.*, 1988). Similarly, giant hogweed (*Heracleum mantegazzianum*) and cow parsnip (*Heracleum* spp.) are also known to cause photosensitivity in Europe and North America, respectively (Knight and Walter, 2003).

Secondary photosensitization is primarily caused by a variety of compounds toxic to the liver that are found in plants, the most important of which are the pyrrolizidine alkaloids (PAs). There are four important plant genera, namely, *Senecio*, *Crotolaria*, *Cynoglossum* and *Amsinckia*, which cause liver disease and secondary photosensitization in North America (Table 21.1). Most PA poisoning of livestock in the western United States is attributable to three species of *Senecio*: tansy ragwort (*S. jacobaea*), threadleaf or wooly groundsel (*S. douglasii* var. *longilobus*) (Johnson and Molyneux, 1984; Johnson *et al.*, 1989) and Riddell's groundsel (*S. riddellii*) (Johnson *et al.*, 1985; Molyneux *et al.*, 1991). *Senecio* species are also the most common cause of PA poisoning throughout the world (Lombardo de Barros *et al.*, 1992; Odriozola *et al.*, 1994). Fiddleneck (*Amsinckia intermedia*), and hound's tongue (*Cynoglossum officinale*), members of the Boraginaceae, have significant quantities of PA capable of producing secondary photosensitization in cattle and horses (John *et al.*, 1974; Stegelmeier *et al.*, 1994). The PA content of plants varies considerably, generally increasing with maturation of the plant and reaching a maximum just before the flower buds open (Candrian *et al.*, 1984). Flowers have the greatest amount of the alkaloid, while seeds of *Crotolaria* and *Amsinckia* concentrate high levels of PA (Johnson and Smart, 1983; Nobre *et al.*, 1994). Pigs

are the most susceptible to PA poisoning, followed by poultry, cattle, horses, goats and sheep (Mattocks, 1968; Craig *et al.*, 1992).

A variety of plant toxins other than PAs are also involved in secondary photosensitization. Among these, horse brush (*Tetradymia glabrata* and *T. canescens*) has been attributed to cause secondary photosensitivity in sheep in North America (Johnson, 1974; Flemming *et al.*, 1922). However, sheep are much more susceptible to horse brush photosensitivity if they concurrently browse on black sage (*Artemisia nigra*), big sage (*A. tridentata*) or both (Flemming *et al.*, 1922; Johnson, 1974). These plants frequently grow in the same locations in western range-lands, and when eaten together have a synergistic effect in causing photosensitivity. Certain plant species like *Lantana camara*, *Agave lecheguilla*, *Tribulus terrestris* and *Panicum* grass species cause secondary photosensitization through inflammation and obstruction of the biliary system (Knight and Walter, 2003). These photosensitizing plants contain saponins that cause inflammation and obstruction of the bile ducts. When the liver cannot excrete bile normally, photosensitizing compounds will accumulate in the blood of animals and will result in photosensitization (Radostits *et al.*, 1994). Sheep eating puncture vine (*Tribulus terrestris*) develop photosensitivity secondary to biliary obstruction that was initially thought to be caused by a mycotoxin, but later found to be the result of steroidal saponins in the plant (Glasonbury and Doughty, 1984; Kellerman *et al.*, 1994). An outbreak of photosensitization in sheep in Great Britain and western Europe due to bog asphodel (*Nartheicum ossifragum*) was caused by plant saponin that occluded the biliary system (Pass, 1987; Burrows, 1990). Alsike clover (*Trifolium hybridum*) is a perennial legume that is commonly grown for livestock consumption in northern parts of North America. This plant can cause secondary photosensitivity in horses that can be attributed to the accumulation of phyloerythrin in the horse's circulation as a result of liver failure (Nation, 1991; Colon *et al.*, 1996).

Animals can also develop photosensitivity while eating moldy grains containing hepatotoxic mycotoxins (aflatoxins) produced by fungi belonging to the genera of *Aspergillus* and *Penicillium*. Moldy straw and water-damaged alfalfa hay may also cause photosensitivity in cattle as a result of mycotoxins that induce hepatitis and cholangitis (Richard, 1973; Putnam *et al.*, 1986; Scruggs and Blue, 1994). Feeding moldy alfalfa hay and silage to cattle has been associated with a secondary photosensitivity due to liver toxins produced by a variety of fungi cultured from the hay (House *et al.*, 1996). However, cattle appear to be more susceptible to these unidentified toxins as compared to sheep, goats and horses (Casteel *et al.*, 1994).

Blue-green algae or cyanobacteria, reported to cause poisoning in North America, include members of the

genera *Microcystis*, *Anabaena* and *Aphanizomenon*. These organisms release potent toxins into the water that can cause severe poisoning in animals drinking the contaminated water. Some of these toxins can act on the liver to cause liver failure and photosensitization (Beasley *et al.*, 1989a, b; Osweiler *et al.*, 1985a; Hoover and Smith, 1995). Southdown sheep may also develop photosensitivity due to a congenital defect in the liver's ability to excrete the photoreactive compound phyloerythrin (Hancock, 1950). In addition to plants, certain drugs and chemicals such as phenothiazine sulfoxide, some tetracycline antibiotics and psoralen, which employs this toxic property as a mechanism of its therapeutic effect to treat conditions such as psoriasis, also produce photosensitivity in animals.

Some molds are not only photosensitizing, but may be toxic to the skin of animals directly or indirectly by other mechanisms. Ergot alkaloids can induce gangrene in all species of animals if ingested over a period of several days or weeks. The most commonly involved fungus is *Claviceps purpurea*. The distal extremities, tail, ears and nose may slough away during this disease. Other mold toxins like serotoxins (trichothecene toxin T-2) have the ability to irritate the skin and mucous membranes of animals on contact. Stomatitis, oral ulcers and necrosis of areas of skin that are in direct contact with moldy plant may be seen in the affected animals. Another trichothecene toxin produced by *Fusarium tricinctum* is diacetoxyscirpenol. It may cause dermal necrosis and gangrene in cattle fed on moldy corn. Acute fescue (a common pasture grass in United States) toxicosis in cattle resembles gangrenous ergotism. In this condition, animals can develop dry gangrene of the extremities. The clavicipetaceous fungi are mostly present in fescue pasture. Fescue hay and seed infected with this mold are reported to be toxic to cattle (Jackson *et al.*, 1984).

## TOPICAL CHEMICALS CAUSING DERMAL TOXICITY IN ANIMALS

Skin is the second most frequent route by which chemicals enter the body of animals. Liquid chemicals are generally absorbed well through the skin if they can partition into the SC lipids. Chemicals in the forms of solids, gases and vapors are only absorbed through the skin if they are first dissolved in the moist layer at the surface of the skin. The skin is a major target for gaseous and liquid pollutants. Various allergic or inflammatory conditions of skin including eczema, atopic dermatitis or acne are often observed on exposure to various chemicals (Baudouin *et al.*, 2002). The topical chemicals that react most specifically with the skin are PAHs, VOCs,

pesticides and heavy metals. A great deal of research has been conducted on experimental exposures to the common laboratory animals as components of mechanistic carcinogenesis studies or as a component of human risk assessments. Unfortunately, less is known about mechanisms of actions of compounds of veterinary importance.

## PAHs

PAHs including benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a,j]acridine, dibenz[a,h]acridine, dibenz[a,h]-anthracene, -dibenzo[c,g]carbazole, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, dibenzo[a,l]pyrene, indeno[1,2,3-cd]pyrene and 5-methylchrysene are reported to cause toxicity in experimental animals (IARC, 1987). Most of these PAHs have been studied in biomedical, biochemical and other laboratory-based experiments. Out of these 15 PAHs, at least eight are present in coal tar that is used as a fuel in the steel industry and blast furnaces. Coal tar is also used in the treatment of skin diseases like dermatitis, psoriasis and eczema. Coal tar can cause allergic or irritant contact dermatitis, erythema, stinging, precipitation of exfoliative dermatitis or generalized pustular psoriasis, tar folliculitis, atrophy, telangiectasia, pigmentation, cutaneous horns, keratoacanthomas and tar warts (Andrew and Moses, 1985; Burden *et al.*, 1994). Coal tar pitch is used for roofing, surface coatings and a variety of other applications (IARC, 1983); three out of these 15 PAHs are found in bitumen and asphalt that are used for paving roads, water proofing and coating pipes. Therefore, considerable risk exists for the animals to come in contact with these toxic PAHs in these localities/applications. Animals are exposed to PAHs through inhalation, eating fodders grown on contaminated soils or through skin when in contact with PAH-contaminated soil or products such as heavy oils, coal tar and roofing tar or creosote (a wood preservative). The toxic levels of PAH have been measured in various tissues of fish that are supposed to be absorbed through the skin (Oliveira-Ribeiro *et al.*, 2005).

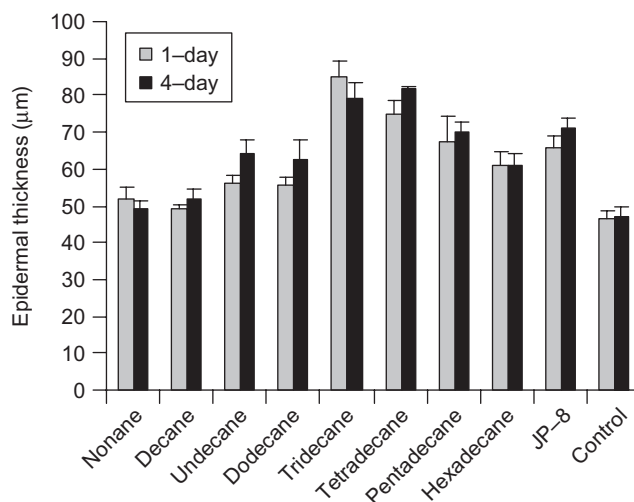
When administered topically, benz[a]anthracene and benzo[j]fluoranthene induced skin papillomas in mice while benzo[k]fluoranthene was active as an initiator of skin tumors in female mice (IARC, 1983). Benzo[a]pyrene induced skin papillomas and carcinomas in mice, rats, guinea pigs and rabbits. Dibenz[a,j]acridine has also been reported to cause skin tumors in mice. Whereas dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene and 5-methylchrysene produced skin tumors in mice when applied topically (IARC,

1973). To date, dibenzo[*a,i*]pyrene has been found to be the strongest tumor-initiating PAH ever tested in rodent skin and mammary tumor models (Mahadevan *et al.*, 2005). However, the tumor promoting potential of benzo[*a*]pyrene can be increased many folds in combination with other toxic chemicals. For example, the combinations of benzo[*a*]pyrene and UV-A light (Wang *et al.*, 2005), or benzo[*a*]pyrene and arsenic (Fischer *et al.*, 2005) treatment synergistically increased tumor incidence and multiplicity in hairless mouse skin. It has been reported that cytochrome P450 1B1 determines susceptibility to dibenzo[*a*]pyrene-induced tumor formation (Buters *et al.*, 2002). However, the organic extracts of black raspberries can inhibit benzo[*a*]pyrene-induced cell transformation *in vitro* possibly by inhibiting the transactivation of activated protein-1 and nuclear factor kappa-B (Huang *et al.*, 2002).

Chlorinated PAHs such as polychlorodibenzodioxines and polychlorofuranes are found in impurities in certain pesticides or as secondary residues in industrial processes. These halogenated PAHs are very toxic for the skin and can cause chloride acne (Baudouin *et al.*, 2002). In a recent incident, former Ukraine Prime Minister and Presidential Candidate Victor Yushchenko developed chloracne from dioxin poisoning. This mysterious poisoning had left his face pockmarked and ashen.

Skin can act as a storage organ for certain halogenated PAHs like hexachlorobiphenyl (Di Francesco *et al.*, 1988). The halogenated PAHs are oily compounds, which can obstruct the pores of the skin, thus resulting in accumulation of sebum and keratin, leading to the formation of cysts. This hypothesis was supported with hyperkeratinization observed around the follicles along with involution of the sebaceous glands by topically applied 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to mouse skin (Panteleyev *et al.*, 1997).

The skin is particularly active in the metabolism of PAHs. Some PAHs are activated by epidermal cytochrome P450 and hydrolases to generate epoxides and diols, active metabolites able to bind to DNA and initiate the processes of carcinogenesis. Other PAHs cause the skin toxicity by different mechanisms. For example, topical application of 7H-dibenzo[*c,g*]carbazole caused the appearance of DNA adducts in mouse skin (Schurdak and Randerath, 1989). Another study exhibited the potentiating effect of iron on 7,12-dimethylbenz[*a*]anthracene in the mouse skin. In addition to genotoxic effects of this PAH, the resulting oxidative stress makes this association tumorigenic in the skin tissue (Rezazadeh and Athar, 1997). The photoactivation of certain PAHs like 2-nitrofluorene by UV radiations in rats can generate intermediates that can bind covalently to RNA and to the skin proteins. This association of 2-nitrofluorene and UV-A is mutagenic and could cause skin cancers (Wierckx *et al.*, 1992).



**FIGURE 21.1** Epidermal thickness (mean + SEM) after 1 and 4 days of exposure to *in vivo* JP-8 or constituent aliphatic hydrocarbons in pigs. Note the maximum toxic *in vivo* response with tridecane (C-13) and tetradecane (C-14) hydrocarbons.

## VOCs

VOCs such as hydrocarbons, ketones, aldehydes, solvents (benzene, fluorocarbons) and gases (methane) are generated from automobiles and industries. Certain VOCs such as benzene are highly genotoxic, inducing cancers in various tissues. Other VOCs can cause precancerous lesions in the lungs and in the skin (Michielsen *et al.*, 1999). Some VOCs can produce inflammatory mediators in cultured epidermal keratinocytes. These mediators such as interleukin-8 and -1 $\beta$  are significantly increased and could favor the allergic/inflammatory reactions as in atopic dermatitis or eczema (Ushio *et al.*, 1999).

Certain VOCs present in jet fuel like ethyl benzene, o-xylene and naphthalene did not produce direct skin irritation in pigskin (Muhammad *et al.*, 2005a). Interestingly, these three hydrocarbons had higher absorption through pigskin compared to other aromatic and aliphatic hydrocarbons studied. Transmission electron microscope and Fourier transform infrared spectroscopy indicated that these PAHs have the potential to extract the lipids out of the SC of skin thus affecting the skin's permeability barrier and increasing the absorption of exogenous substances (Muhammad *et al.*, 2005b). On the other hand, less volatile hydrocarbons of jet fuels such as tridecane, tetradecane and pentadecane produced significant direct dermal irritation and microscopic changes in pigskin (Figure 21.1).

Polychlorinated naphthalenes are present in lubrication greases, oils, fuels and in wood preservatives. Chlorinated naphthalenes can cause severe

hyperkeratosis in cattle. The most prominent lesions were observed on the neck and wither but may affect the entire integument. This dermal toxicity in cattle needs to be differentiated from avitaminosis A. Chlorinated naphthalenes did not produce hyperkeratosis in sheep and swine (Clarke and Clarke, 1975). Ethylene glycol monomethyl ether (EGME) and its acetate ester (EGMEA) are moderately volatile liquids with very good solubility properties. They are used in paints, lacquers, stains, inks and surface coatings and as an anti-icing additive in hydraulic fluids and jet fuel. EGME and EGMEA are efficiently absorbed via dermal penetration. Skin absorption in animals may contribute substantially to the total uptake following skin contact with liquids or vapors containing EGME or EGMEA (Johanson, 2000).

## PESTICIDES

The word “pesticide” in this chapter will encompass various relevant chemicals including insecticide for controlling termites or fleas, herbicides for ridding the crops/lawn of dandelions or fungicides that protect plants during fungal diseases. Despite oral ingestion, dermal absorption is the major source of pesticide exposure in occupational, agricultural and veterinary settings, a topic recently reviewed (Riviere, 2006). Since most of the pesticides are highly lipophilic, their penetration and accumulation in skin with resulting local dermal irritation and/or mild to severe systemic toxic effects in the exposed animals cannot be overlooked.

Livestock and pet animal exposure to pesticides is usually accidental. The most common source of dermal toxicity induced with insecticides in both large and small animals is the miscalculation of concentrations for spraying and dipping procedures. A misplaced decimal point can result in hundred- or thousand-fold increase in exposure. A common source of non-recommended exposure to animals is the inadvertent mixing of powdered pesticides, mistaken for salt or mineral preparations, into animal feeds. A low-level contamination of animals by insecticides can also occur with the use of persistent chlorinated hydrocarbons such as dichlorodiphenyl-trichloroethane (DDT), aldrin, dieldrin and heptachlor, etc. on crops which may be used for animal feeds. These insecticides may persist in soil for several years. Thus, forage crops grown on such soils could be sufficiently contaminated to produce toxic effects in animals. Other sources of insecticide exposure in animals include carelessness in leaving insecticides accessible to animals, leaving insecticide preparations on sills of sheds or barns, inadequate covers on back rubbers containing insecticide concentrates, or using pesticide containers to

water and feed animals without proper decontamination (Osweiler *et al.*, 1985b). Thus, animals can exhibit signs of dermal toxicity after oral or systemic exposure to pesticides. On the other hand, washing the skin of humans or animals after exposure to a pesticide or other chemical may leave a major portion of the dose on/in the washed skin. This skin residue can contribute to the toxicity of a pesticide by continued post-wash absorption (Zendzian, 2003). According to recent statistics on occupational dermatosis among private farmers, pesticides are the causative factor in 18% of skin disorders (Spiewak, 2003). The other most frequently identified causative factors for occupational dermatosis were plant dusts (38%), animal allergens (36%), metals (29%) and rubber chemicals (15%).

Certain organophosphorus insecticides such as chlorpyrifos have been shown to exert their systemic effects in the form of cholinesterase inhibition after dermal application to rats (Abu-Qure *et al.*, 2001). The topical application of chlorpyrifos and cypermethrin to rats showed an inhibition of acetylcholinesterase and butyrylcholinesterase activity in the brain (Latuszynska *et al.*, 2001). Another study suggested that skin could act as a reservoir and may release chlorpyrifos over a prolonged period (Griffin *et al.*, 2000). Pyrethrins are insecticides that are derived from the extract of chrysanthemum flowers. The synthetic forms of pyrethrins are called pyrethroids. The initial plant extract (crude pyrethrum) contains about 30–35% pyrethrins. Allergic contact dermatitis has been observed in sensitized and non-sensitized guinea pigs by topical exposure to various extracts from pyrethrum flowers (Rickett *et al.*, 1972). Occupationally, skin is the main route of pyrethroid absorption. The major toxic effect of dermal exposure is paraesthesiae, presumably due to hyperactivity of cutaneous sensory nerve fibers (Bradberry *et al.*, 2005). The face is most commonly affected and the paraesthesiae are exacerbated by sensory stimulation such as sunlight, scratching, heat, sweating or water application. The specific treatment is not generally required, as paraesthesiae usually resolve in 12–24 h. Topical application of dl- $\alpha$  tocopherol acetate (vitamin E) may reduce the severity (Bradberry *et al.*, 2005). Similarly, cypermethrin (a pyrethroid) can penetrate through the skin of animals and can exert its systemic effects (Latuszynska *et al.*, 2001). Dermal exposure to permethrin (another pyrethroid) has often been considered a mitigating factor in Gulf War Syndrome (Riviere *et al.*, 2002). Studies have shown that systemic exposure of certain chemicals such as diisopropyl fluorophosphate (DFP) or pyridostigmine bromide and topical application of DFP or *N,N*-diethyl-*m*-toluamide (DEET) can alter the disposition of [<sup>14</sup>C] permethrin in skin and possibly its bioavailability in soldiers simultaneously exposed to these chemicals (Baynes *et al.*, 2002a).



A number of chemicals are used as fumigants for control of insects, nematodes, fungi and weeds in soils, stored grains, feedstuffs, storage spaces in mills and common carrier vehicles. Most of the toxicology of such chemicals is studied in laboratory animals and needs to be extrapolated for the domestic animals. One such chemical, ethylene dichloride, is reported as irritating to the skin and mucous membranes. It can be absorbed through the skin although large doses are required to cause toxicosis in animals. Ethylene dibromide and metham-sodium are other skin irritants that also have the potential to absorb through the skin. Fluoroacetate was developed for insecticide and rodent control applications in early 1940s. This chemical has been reported to absorb through the abraded skin but not through the intact skin (Osweiler *et al.*, 1985b). Paraquat is a non-volatile bipyridyl herbicide and is insoluble in water. Low-to-moderate amounts of bipyridyl herbicides are absorbed from the skin of animals. Direct contact of animals with paraquat results in irritation to skin (Longstaffe *et al.*, 1981). Experimental studies in pigskin demonstrated epidermal toxicity in the absence of appreciable transdermal absorption (Srikrishna *et al.*, 1992).

Many chemicals and solvents are dermal penetration enhancers. Such chemicals may increase dermal penetration by damaging the skin's barrier, by modifying the SC, or by promoting the partitioning of co-penetrating chemicals (Moser *et al.*, 2001; Medi *et al.*, 2006). This scenario may serve useful purposes in transdermal drug delivery but at the same time penetration enhancers may also increase skin penetration of harmful chemicals (Baynes *et al.*, 2002a). The percutaneous penetration of five pesticides having different solubilities was studied through a slightly damaged skin. It was observed that the percutaneous penetration of the most hydrophilic compounds will be affected most, and may significantly affect the rate, lag-time as well as total penetration of chemicals (Nielsen, 2005). A combination of two solvents, pyridostigmine bromide and DFP, increased the dermal absorption of DEET through isolated perfused porcine skin (Riviere *et al.*, 2003). Earlier studies by Baynes *et al.* (1997) reported that the extent of DEET absorption was greater with dimethyl sulfoxide (DMSO) and acetone than with ethanol in rat and mouse skin. These studies support that DEET can have sufficient systemic exposure to potentially cause signs of toxicity when simultaneously applied with pesticides such as permethrin or carbaryl. Thus, penetration enhancement can have undesirable consequences. In this context, commercial sunscreen formulations are found to significantly increase the transdermal penetration of a herbicide 2,4-dichlorophenoxyacetic acid in *in vitro* rat skin (Pont *et al.*, 2004). In addition to the active ingredients, "inert" ingredients in sunscreen or pesticide formulations may also be responsible for controlling the transdermal

penetration of herbicide 2,4-dichlorophenoxyacetic acid (Pont *et al.*, 2003) and carbamate insecticide carbaryl (Baynes and Riviere, 1998), respectively.

Pentachlorophenol (PCP) has been widely used as a pesticide. The persistence of PCP in soil and water and apparent widespread use has resulted in significant exposure to animals. Young swine have died following dermal exposure to freshly PCP treated wood used in farrowing crates or farrowing houses. *In vivo* studies in swine demonstrated that exposure to PCP-contaminated soil can result in significant dermal absorption of the pesticide (Wester *et al.*, 1993; Qiao *et al.*, 1997). It has been demonstrated that the presence of solvent and/or surfactant mixtures can influence PCP absorption in the isolated, perfused porcine skin flap (IPPSF) (Riviere *et al.*, 2001). Baynes *et al.* (2002b) demonstrated that PCP is fairly well absorbed across porcine skin. PCP absorption in skin was greater in water or water-based mixtures than in 100% ethanol. PCP is barely soluble in water, but this pesticide is more likely to partition from water than an organic solvent vehicle such as ethanol into the SC. This situation warrants the dermal uptake of lipophilic pesticides through the skin of animals as the latter have free access to water all the time. Both PCP and its major metabolite tetrachlorohydroquinone (TCHQ) could induce mice skin epidermal hyperplasia and proliferating cell nuclear antigen (PCNA) labeling index in the epidermis. However, TCHQ caused a more significant induction of epidermal hyperplasia and PCNA positive cells than PCP. Furthermore, topical application of PCP induced significant organ enlargement and lymphoma in mice (Chang *et al.*, 2003).

The insecticide D-limonene toxicosis has been rarely described in canine species. One study has reported skin lesions consisting of coalescing erythematous patches in the cat groomed previously with a D-limonene-based insecticidal shampoo. Dermatohistopathologic changes included multifocal areas of acute coagulative epidermal necrosis (Lee *et al.*, 2002). Epichlorohydrin (ECH) is one of the more commercially important aliphatic epoxides used extensively as an insecticide, an industrial intermediate and as a laboratory reagent. It is a volatile, colorless liquid and behaves as an alkylating agent. Reports have shown that it causes dermal toxicity in animals (Giri, 1997). Dodecen-1-ol (an insect attractant) acted as a primary skin irritant, since it caused superficial chemical burns (Beroza *et al.*, 1975). Ortho-phenylphenol (OPP) and its sodium (SOPP) and potassium (POPP) salts are used as fungicides and disinfectants. OPP has been reported as irritating, and SOPP and POPP as corrosive for the skin and mucous membranes (Bomhard *et al.*, 2002).

White phosphorus had been used to prepare rodenticides that are generally mixed with a greasy or oily base. Skin irritation or burning may occur from dermal

exposure to high concentrations of such rodenticides but absorption is not observed with dermal exposure. Thallium is another rodenticide that is primarily used by government agencies. In subacute thallium poisoning in dogs and cats, reddening and pustule formation in the skin starting from the ears and nose and progressing to the axilla, abdomen and the rest of the body, associated with loss of hair, was reported. In chronic thallium poisoning, skin hyperkeratosis, parakeratosis, hyperemia and vivid discoloration of skin have been observed in the cat (Osweiler *et al.*, 1985b).

## DETERGENTS, SOLVENTS, CORROSIVES AND OTHER HOUSEHOLD PREPARATIONS

Several daily use products (soaps, detergents, drain cleaners; acids, alkalis, etc.) available in homes and other work places may cause dermal irritation. There is scarce information in literature regarding toxicosis due to these daily use products in animals. We will discuss briefly the possibilities of dermal irritation in animals based on well-known scientific principles and published reports.

The evaluation of cleansing products depends upon their cleansing properties (detergent or adsorptive), on the rinsability of the product, on the amount and nature of the additives and on the general and specifically epidermal toxicity of the components (Raab, 1990). In this context, one such preparation is denture cleaner in which the major toxic component is sodium perborate which decomposes to form hydrogen peroxide and sodium borate. These products are strongly alkaline, very irritating to the skin and mucous membranes, and thus can produce dermal toxicity in exposed animals. Similarly, constant or repeated exposure of skin to anionic detergents (sulfomated or phosphorylated hydrocarbons) causes irritation with the removal of natural oils and can result in thickening of skin along with weeping, cracking, scaling and blistering (Dreisbach, 1983). There is evidence that detergent substances are important components of cleansing materials, but their detergent cleansing action may also result in skin toxicity (Marks and Dykes, 1990). Non-ionic detergents such as alkyl and aryl polyether sulfates, alcohols or sulfonates are comparatively less irritating than ionic ones. The acute skin irritation potential of various detergent formulations can be assessed with a patch test (Robinson *et al.*, 2005). In this test, the time of exposure required for 50% of subjects to show a positive skin reaction ( $TR_{50}$  value) is calculated for each product. Using this approach, 24 detergent preparations were tested in seven individual studies. The dermal irritation profiles

could be categorized as follows (by decreasing irritancy): mold/mildew removers (average  $TR_{50} < 0.37h \geq$  disinfectants/sanitizers (0.64h) > fabric softener concentrate (1.09h) < aluminum wash (1.20h) > 20% SDS (1.81h)  $\geq$  liquid laundry detergents (3.48h)  $\geq$  liquid dish detergents (4.16h) < liquid fabric softeners (4.56h) < liquid hand soaps (4.58h) < shampoos (5.40h) = hard surface cleaners (6.34h) > powder automatic dish detergents (>16h) < powder laundry detergents (>16h) (Robinson *et al.*, 2005). Cleansing can also be accomplished with non-detergent containing cleaners (Marks and Dykes, 1990). Drain cleaners consisting of high concentrations (25–36%) of sodium hydroxide and sodium hypochlorite are extremely toxic and caustic to the skin. Direct skin contact to these agents causes coagulative to liquefaction necrosis by dissolving proteins and saponifying lipids. Soaps have been used for thousands of years as part of daily life; however, they are limited by their irritancy to the skin due to their alkaline nature and their tendency to form insoluble and inactive salts when combined with either hard water or sea water (Kirsner and Froelich, 1998).

Calcium cyanide, which is a special constituent of fertilizers, may act as a contact irritant causing skin ulcers. Chlorobromomethane (a fire extinguisher liquid) causes intense skin irritation from exposure to either liquid or its vapors, although this chemical has low dermal absorption. Skin contact to another chemical methyl bromide (used in fire extinguishers, refrigerants or fumigants) can result in irritation and vesiculation. Clinical poisoning may occur from its skin absorption (Osweiler *et al.*, 1985b). Liquid soldering fluxes mainly contain zinc chloride and a high proportion of hydrochloric acid. These components are caustic or corrosive and acute exposure results in direct irritation to skin. Glues and adhesives are often supplied in hydrocarbon solvents. Dermal exposure to these preparations may also produce irritation. The corrosive activity of hypochlorite (a common component of laundry bleaches) on skin is due to oxidizing potency or available chlorine. Alkalinity of some preparations may contribute to tissue injury. Metal cleaners and oven cleaners contain components like soda/potash and KOH/NaOH in high concentrations, respectively, in addition to petroleum-based solvents. All these components would be expected to cause dermal irritation. Ethylene glycol and propylene glycol (PG) are used in most of the topical pharmaceutical and cosmetic preparations as solvents. The localized dermal effects from both solvents are mild, while the published data suggested that PG might have a skin contact sensitization potential (Lakind *et al.*, 1999).

The alkalis present in oven cleaners may cause severe necrotic lesions similar to drain cleaners. Domestic animals, especially pets, may be exposed dermally to paints and varnish removers containing mixtures of

benzene, methanol, acetone and toluene. These solvents can absorb through the skin in considerable amounts. Certain perfumes can cause local irritation of skin. This is probably due to alcohol serving as the primary vehicle. Rubbing alcohol (ethyl alcohol) may sometimes cause cutaneous hyperemia. Rust removers whose major toxic components are hydrochloric acid, phosphoric acid, hydrofluoric acid, etc. can have direct corrosive and necrotizing action like other acids. Most shampoos would not be expected to cause severe dermal irritation. Generally, skin exposures to acids, alkalis and phenols may lead to lesions that can vary from mild dermatitis to severe corrosion of the skin.

## SYSTEMIC COMPOUNDS CAUSING DERMAL TOXICITY IN ANIMALS

### Heavy metals

Metals are intrinsic in nature and are utilized by animals for many essential functions. As a rule, metals are required for life and health in small quantities but are toxic in excessive amounts. The toxic heavy metals in animals include arsenic, lead, cadmium, chromium and nickel. These metals contaminate the environment from industrial, automobile and pesticide activities. The moderately toxic metals are iron, zinc, selenium, mercury and copper. Redistribution of metals in the environment is mainly responsible for access of animals to toxic metals not normally assessable. Metal toxicosis in animals results from the systemic administration of metals. The mechanisms involved in the toxicity of certain metals like cadmium, chromium and nickel have indicated that these metals act directly or indirectly on intracellular proteins in the skin (Carlisle *et al.*, 2001). Inorganic arsenic exposure in drinking water is linked to skin, bladder and lung cancer. The mechanism of arsenic-induced cancer is not clear, but it is proposed that arsenic can generate reactive oxygen species, suggesting that oxidation of DNA may play a role in carcinogenesis (Fischer *et al.*, 2005).

Heavy metals can form reversible complexes with organic ligands during the chelation process. For example, lead may be concentrated as a complex in bile. Proteins such as metallothionein serve as transport molecules for metals (Hammond and Beliles, 1980). This scenario may alter the availability and delivery of metals to specific organs. On the other hand, certain toxic metals can accumulate in definite tissue locations. For instance, bone which can store high concentrations of lead can result in distribution to plasma protein or skin and hair. Susceptibility to dermal absorption of metals may be increased when the metal is organically bound, such as

methyl mercury or tetraethyl lead. One such study indicated that the dermal symptoms of inorganic, aryl and methoxy ethyl forms of mercury in cattle include skin pustules, ulcers, depilation (starting at the root of tail) and skin keratinization. Animals with severe skin damage are severely ill. The febrile response and skin color changes occurring in mercury poisoning can easily be confused with hog cholera and erysipelas. At the start of clinical syndrome, mercury poisoning in pigs also resembles the early skin reddening and abnormal gait resulting in organic arsenic poisoning (Osweiler *et al.*, 1985b).

Hair of animals may be the most common site of metal depositions since hair has a chemical affinity for heavy metals like arsenic, lead, thallium, selenium, bismuth and mercury. These metals have the potential to react with sulfhydryl groups of amino acids, particularly cysteine, in the follicular proteins and incorporated into the keratin. Because of the relative ease of getting hair samples, concentrations of metals in hair may be used as a biomarker for risk assessment surveys to determine the chronic heavy metal exposure in animals.

Forage crops grown on seleniferous soils contain sufficient amounts of selenium to cause poisoning in domestic animals. Selenium may cause acute and chronic poisoning in all animals, especially cattle, sheep, horses, swine, chicken and dogs. A loss of hair/wool, deformation and sloughing of the hoof are seen in cattle, sheep and horses that were poisoned chronically with selenium. This condition is commonly known as alkali disease; a misnomer (Osweiler *et al.*, 1985b). Chronic selenium (Se) toxicosis has been diagnosed in pigs with loss of hair, necrotic areas in the skin and lesions of the coronary band and hooves. The cause of this intoxication was the addition of the calculated amounts of sodium selenite directly to the feedstuff instead to mineral premix (Mihailovic *et al.*, 1992). In one occurrence of arsenic poisoning in cattle, arsenic in the hair of affected survivors was assayed at 0.8–3.40 ppm versus 0.09–0.10 ppm in randomly selected control samples (Riviere *et al.*, 1981). Inorganic tin salts are poorly absorbed and rapidly excreted in the feces; as a result they have a low toxicity. The main results of inorganic tin toxicity are skin and eye irritation; cholangitis of the lower biliary tract, and later hepatotoxicity; and neurotoxicity (Winship, 1988). Similarly, considerable skin changes in the form of scaliness on the dorsum of the neck and sloughing of the epidermis over neck, withers, ears, briskets, shoulders, back and tail were observed in beef calves after systemic administration of iodine. A slight degree of hair loss was also reported (Osweiler *et al.*, 1985b).

Chromium is an essential trace element required for normal protein, fat and carbohydrate metabolism. It also helps in energy production and increasing lean body mass. A unique form of bioavailable chromium is

niacin-bound chromium (NBC) that is used to promote a healthy lipid profile. The primary skin irritation test with NBC on New Zealand albino rabbits indicated that NBC was slightly irritating (Shara *et al.*, 2005).

Compounds other than metals can also produce dermal irritation on systemic administration. Oral administration of benzo[a]pyrene (PAH) caused the formation of DNA adducts in the skin (Baudouin *et al.*, 2002). The ingestion of hexachlorobenzene (VOC) in the rat leads to the formation of precancerous lesions in the skin and lungs (Michielsen *et al.*, 1999). The high doses of pyrethrins (insecticides) fed to rats can induce benign skin lesions (USEPA, 1995). Polybrominated biphenyls were used as fire retardant in many parts of the world. On accidental ingestion of this chemical with contaminated feed or by licking the contaminated wood, animals with alopecia and thickened skin over the thorax, neck and shoulder had been noted. Systemic exposure of ruminants and sheep to thiocarbamate (herbicide) can induce alopecia (Osweiler *et al.*, 1985b). Severe dermatitis over the ventral portion of the abdomen and inner surfaces of the limbs was developed in pigs that were given adriamycin (a systemic antibiotic) intravenously (Van Vleet *et al.*, 1979).

## ASSESSMENT METHODS

*In vivo* testing of certain chemicals for their irritating or sensitizing potentials and assessment of their dermal uptake are other sources of skin toxicity in experimental animals. Animal welfare regulations mandate the careful monitoring of dermal irritation and toxicity studies due to the potential for causing pain and skin damage resulting from cutaneously applied materials. In the U.S., the care and use of rabbits, guinea pigs, minipigs, dogs and non-human primates are regulated by the U.S. Department of Agriculture under the Animal Welfare Act (AWA, amended, 1985). Rats and mice are not included in this act, yet most institutions use these animals under similar internal standards for these species. According to this act skin irritancy testing is a procedure which can cause slight pain in the animal. Severe erythema, erosions, ulcers, abscesses and necrosis are the painful skin reactions produced in response to topical application of chemicals. The degree of skin toxicity in experimental animals varies with response to acute, subchronic or chronic dermal irritation studies.

Among assessment methods capable of causing dermal toxicity, cutaneous microdialysis, tape stripping and skin surface biopsies are the major *in vivo* models used to assess the dermal uptake of topically applied toxicants. Microdialysis is a promising technique for determination of *in vivo* dermal absorption (Simonsen *et al.*, 2004).

This technique consists of a semipermeable membrane forming a thin hollow tube that functionally resembles a blood vessel. The microdialysis probe is implanted in the dermis of the skin via a guide cannula. This technique is minimally invasive, and produces a minor trauma during insertion of the guide cannula for the implantation of the microdialysis probe; although this trauma is reversible (Groth and Serup, 1998) this can damage the skin considerably in sensitive animals. After the insertion of probe, an equilibration period of minimum 90 and 30 min in human and rat skin, respectively, is necessary to allow the effects of trauma to diminish (Groth, 1996). Tape stripping is suitable for studying the penetration of topically applied chemicals into the SC. This is composed of serial stripping of SC with the help of common adhesive tapes. This technique is invasive to the minimal extent and frequently used to study the reservoir effect of the SC for topically applied constituents and the assessment of their *in vivo* percutaneous penetration effects (Muhammad and Riviere, 2006). Another minimally invasive technique is skin surface biopsies. With the help of this technique, it is possible to track the permeation of a compound through the SC by taking consecutive biopsies in the same area (Dykes *et al.*, 1997).

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## Blood and bone marrow toxicity

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### INTRODUCTION

Hematotoxicology is the study of adverse effects of drugs, non-therapeutic chemicals and other agents in our environment on blood and blood forming tissues such as bone marrow. Assessment of blood and bone marrow in veterinary toxicology is important since chemicals and drugs can significantly affect the cellular and humoral components of the blood. The hematopoietic system is unique and serves as a “cornerstone” to one’s understanding of the toxicology of blood and bone marrow. Hematopoietic cells are the most dynamic, proliferative and mitotically active cells in an animal. In humans, mature blood cell production is estimated at 1.5 million cells per second (Car, 2010). Therefore, toxicity to circulating blood cells or their precursors may be very serious and can result in life-threatening anemia, hemorrhage or profound infections.

Bone marrow is a common target for a variety of chemotherapeutic agents in animals and humans, some of which can result in total marrow aplasia (Lund, 2000). Many other drugs can also negatively impact blood components; therefore, the evaluation of blood and bone marrow in preclinical drug development is required by many regulatory agencies when assessing the safety and efficacy of test compounds (Bolliger, 2004). Because blood and bone marrow can be relatively easily assessed, sequential or parallel monitoring of blood and bone marrow has become a standard component of hematotoxicity screening by the pharmaceutical industry as well as in the clinics. This chapter is intended to provide a basic introduction to blood and bone marrow as well as illustrate some toxicant-induced changes. Information provided in this chapter should serve as an important and

accessible tool for understanding basic concepts, and for monitoring and characterizing toxic responses of the blood and bone marrow.

### BONE MARROW

Hematopoiesis normally occurs in the bone marrow of both flat bones and long bones, and in mice it can also occur normally in the spleen (Gasper, 2000). Blood supply to the marrow is mainly provided by nutrient arteries that penetrate cortical bone, particularly at the mid-shaft of long bones. These arteries extend along the longitudinal axis of the bone and send off radial branches throughout the marrow. These branches terminate at the periphery of the marrow cavity and connect with venous vessels. Venous or vascular sinuses of marrow are thin-walled vessels with abundant anastomoses, and they carry blood back to central veins. Marrow lacks lymphatic vessels. Nerves in the marrow are vasomotor and found in association with the vasculature.

The hematopoietic compartment of the marrow consists of irregular and anastomosing cords that lie between vascular sinuses (Bloom, 1997). Blood cells are produced in the hematopoietic compartment of the marrow and reach the circulation by migrating through the vascular sinus endothelium. Marrow fat cells represent a mechanical buffer that occupy or release space within the medullary cavity in response to changing demands for hematopoiesis. Adventitial reticular cells provide structural support for hematopoietic colonies. The extent of marrow fat and adventitial coverage of vascular sinuses varies inversely with the degree of hematopoiesis and the



rate of cell delivery to the bloodstream. Thus, the amount of fat in the marrow increases with age (Bloom, 1997).

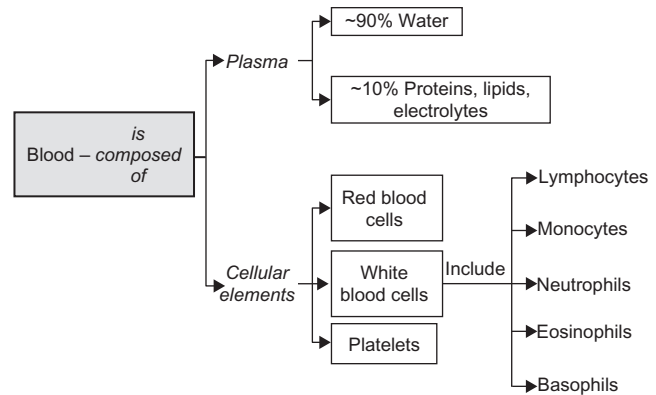
## HEMATOPOIETIC STEM CELLS

A pluripotent stem cell that can produce all blood cell types, including lymphocytes, is said to be totipotent. Most totipotent stem cells in adults are not in the cell cycle, but are quiescent. Totipotent stem cells account for less than 0.2% of the total hematopoietic cells in the marrow. In adult mammals, most of these cells are lodged in the bone marrow, but small numbers enter, circulate and return from blood. Low numbers are also present in the spleen. Totipotent hematopoietic stem cells produce pluripotent myeloid and lymphoid stem cells. Pluripotent stem cells give rise to progenitor cells which are capable of forming colonies in marrow culture, like stem cells, but lack long-term self-renewal capacity and are generally more restricted in their differentiation (Meyer and Harvey, 1998). Many progenitor cells are in the active cell cycle. They are capable of producing progeny (blast cells) that can be recognized morphologically. These resultant blast cells are classified as “precursor cells.”

The pluripotent myeloid stem cells give rise to a series of progressively more differentiated progenitor cells that support the production of all nonlymphoid cells. When grown in an *in vitro* cell culture assay, each colony of cells represents one progenitor cell and is referred to as a colony-forming unit (CFU). With time, a few large colonies may appear in culture, originating from a more immature progenitor cell, and these are referred to as burst-forming units (BFUs) (Meyer and Harvey, 1998). Both the local marrow microenvironment and systemic humoral factors influence hematopoiesis to stimulate or suppress the proliferation and differentiation of single or multiple cell lineages. In general, multi-colony growth factors such as interleukin-3 (IL-3) stimulate early progenitor cells, whereas single colony factors such as erythropoietin (EPO) and granulocyte-macrophage colony stimulating factor (GM-CSF) act on more differentiated progenitor cells such as erythroid and granulocyte-macrophage progenitor cells, respectively.

## BLOOD COMPONENTS

Blood consists of cells and protein-rich plasma (Figure 22.1). Blood cells include erythrocytes (red blood cells), platelets (usually called thrombocytes in nonmammalian species) and leukocytes (white blood cells). The leukocytes in most vertebrates are classified

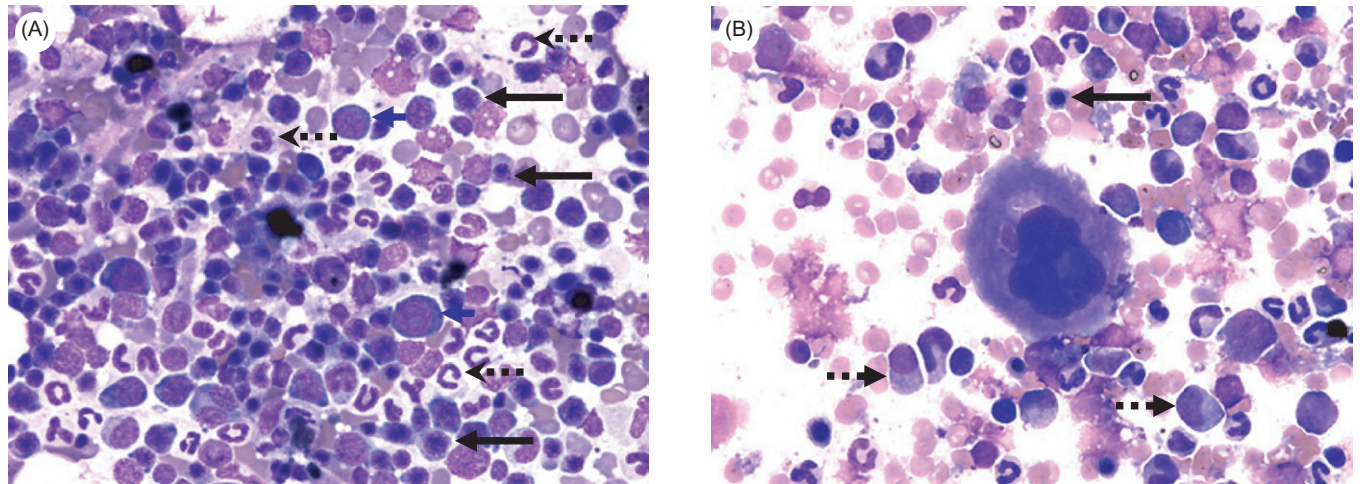


**FIGURE 22.1** Blood components. The blood consists of the fluid-rich plasma component and the cellular component consisting of red and white blood cells. The white blood cells are composed of neutrophils, basophils, eosinophils, monocytes and lymphocytes. See text for details on morphology of these cells.

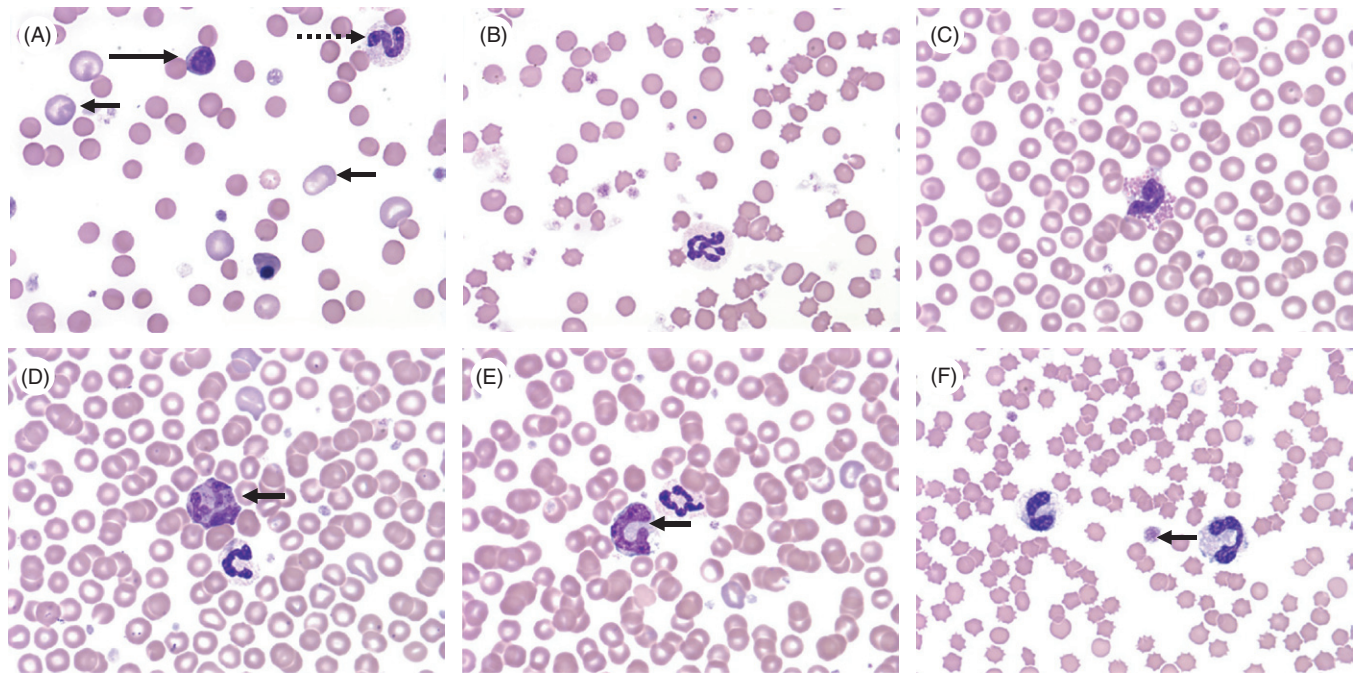
as granulocytes (neutrophils, eosinophils and basophils) or mononuclear cells (monocytes and lymphocytes). Freshly drawn blood rapidly clots, but clotting can be prevented by the addition of an anticoagulant, thereby allowing for assessment of blood cell morphology on a properly prepared blood film (Figures 22.2 and 22.3) and separation of the plasma and cellular components into three distinct layers after centrifugation (Andrews, 1998; Meyer and Harvey, 1998). The lowest layer is red since it consists mostly of erythrocytes, and the percent volume of this layer with respect to the total blood volume determines the packed cell volume (PCV) or hematocrit (HCT), which is normally approximately 35–45% in most species. The thin, gray-white middle layer immediately above the erythrocytes is the buffy coat, and it accounts for approximately 1% of the blood volume. The buffy coat contains platelets and leukocytes, but immature erythrocytes may also be present in this layer. The top (fluid) layer is the plasma. Plasma contains 91–92% water and 8–9% solutes such as proteins, lipids and electrolytes (Figure 22.1). A variety of homeostatic mechanisms ensure that plasma volume, pH and constituents are tightly regulated. When clotting of blood occurs, fibrinogen and many coagulation factors are depleted from the fluid component. The resulting fluid is called serum (Andrews, 1998; Meyer and Harvey, 1998).

### Erythrocyte, leukocyte and platelet formation

Immature blood cell precursors are generally larger than mature forms, and they have a large euchromatic nucleus resulting in a high nuclear to cytoplasmic (N:C) ratio. As blood cells mature, their size and N:C ratio gradually decrease.



**FIGURE 22.2** (A) Examples of erythroid precursors and granulocytic precursors (arrowheads) in the bone marrow of a dog. Diff-quick stain. Early erythroid precursors (arrow heads), late erythroid precursors (solid arrows) and granulocytic precursors (dotted arrows) are shown. (B) A mature megakaryocyte in the bone marrow of a dog is shown in the center of the picture. Erythroid and myeloid precursors are also noted (arrows). Modified Wright's stain.



**FIGURE 22.3** Normal and some abnormal erythrocyte and leukocyte morphologies. (A) Two large basophilic polychromatophilic erythrocytes or reticulocytes (small solid arrow) are present in the blood from a dog with IMHA. A normal neutrophil (dotted arrow), a metarubricyte and a lymphocyte (long arrow) are also present. Modified Wright's stain. (B) Heinz bodies in the blood from a cat appearing as pale "spots" within erythrocytes with a modified Wright's stain. (C) Eosinophil with round granules in the blood from a dog. Modified Wright's stain. (D) Basophil (arrow) and neutrophil in the blood from a dog. Modified Wright's stain. (E) Monocyte (arrow) and neutrophil in the blood from a dog. Modified Wright's stain. (F) Toxic neutrophils in the blood from a cat demonstrating Döhle bodies, cytoplasmic vacuolation and basophilia. A platelet (arrow) is also identified in the field. Modified Wright's stain.

### Erythropoiesis

Erythropoiesis is the process by which committed hematopoietic progenitor cells develop into anucleate, hemoglobin-containing, biconcave discs (in mammals) called

erythrocytes. EPO regulates this process. Rubriblasts, the first recognizable erythroid precursor, undergo approximately four cell divisions to generate the last nucleated stage, the metarubricyte (Figure 22.2a). However, intracellular hemoglobin concentration is an important



factor in determining the number of cell divisions a cell undergoes to produce this stage (Car, 2000). With the extrusion of the nucleus, the resulting immature erythrocyte is termed a reticulocyte. The reticulocyte persists for about 2 days after release into the circulation before maturation into a mature erythrocyte. Under normal situations, few reticulocytes are seen in peripheral blood, but some strains of rodents may normally have up to 10% circulating reticulocytes (Fernandez and Grindem, 2000). The lifespan of mature erythrocytes ranges from 43 to 145 days, depending on the species (Christian, 2010).

### **Granulopoiesis**

Granulopoiesis is the process by which committed hematopoietic progenitor cells develop into granulocytes under the influence of various growth factors and cytokines. Neutrophils and monocytes are derived from a common progenitor, the colony-forming unit granulocyte-macrophage (CFU-GM), whereas eosinophils and basophils are derived from different progenitor cells (CFU-Eo and CFU-Ba, respectively). The first recognizable granulocytic precursor is the myeloblast. Early granulocyte precursors (myeloblast and promyelocyte) appear similar between the various granulocytic cell lines until the myelocyte stage, which is the final stage capable of cell division. At this stage, they develop characteristic secondary lineage-specific granules (neutrophilic, eosinophilic or basophilic). During the next stage (the metamyelocyte), the nucleus of the granulocyte indents and lobulation begins, eventually producing the characteristic polymorphonuclear appearance (Figure 22.2a, b) (Gasper, 2000; Smith, 2000).

### **Platelet formation**

The megakaryocyte is a polyploidy cell with cytoplasm that becomes increasingly granular with maturity (Figure 22.2). Platelets are derived from the megakaryocyte cytoplasm. The mechanism by which platelets are released from their parent cell is unclear, but it appears to be by cytoplasmic fragmentation. This process may take place in an extramedullary location (i.e., lung, spleen). The cytokine thrombopoietin is the major stimulus for megakaryocyte proliferation, platelet production and differentiation from a common progenitor cell, the CFU-GEMM (Andrews, 1998). If unconsumed, the platelet lifespan ranges from 3 to 10 days depending on the species (Weiss, 2000).

## **Erythrocyte, leukocyte and platelet biology**

### **Erythrocyte**

#### *Erythrocyte function*

The function of red blood cells is to transport oxygen to tissues. Decreased hemoglobin lowers oxygen delivery

to tissues. This results in increased EPO secretion from the kidney and subsequent stimulation of erythrocyte production.

#### *Morphology of erythrocytes*

Mature erythrocytes are anucleate biconcave discs. The degree of concavity varies among the domestic species, and typical biconcave erythrocytes, as indicated by central pallor, are present in dogs, cows, sheep, rats and mice. The concavity and resulting pallor are not prominent in horses and cats. In most species, an increase in central pallor indicates decreased hemoglobin content. Slight anisocytosis (variation in size) of erythrocytes is common in most animal species, and poikilocytosis (variation in shape) is normally present on smears made from the blood of goats and some species of deer. Erythrocytes may adhere to each other and form long chains resembling stacks of coins. This phenomenon, called rouleaux formation, is normally prominent in horses and cats, intermediate in dogs and pigs, and rare in ruminants. Rouleaux formation is often increased in inflammatory states. Reticulocytes are immature anucleate erythrocytes that appear large and polychromatophilic (bluish red color) on Wright's stained blood films (Figure 22.3a). Reticulocytes contain residual ribonucleic acid (RNA) (ribosomes and polyribosomes) and mitochondria, which aggregate into a reticular mesh when stained with vital stains (e.g., new methylene blue) but not with Romanowsky-type stains. Quantitation of reticulocytes in circulation is used as an index of bone marrow erythropoietic response since circulating reticulocytes should be numerous when the marrow is responding to an increased demand for erythrocytes. Heinz bodies result from the oxidation and aggregation of hemoglobin and are therefore an indication of oxidative damage (Meyer and Harvey, 1998; Harvey, 2001). They appear as pale red inclusions within or along the edge of the cytoplasm using Romanowsky-type stains, but they stain light to medium blue with reticulocyte stains (Figure 22.3b). Similar to diagnostic medicine, the above alterations in RBC shape or appearance are only a portion of those that may be seen in clinical patients or in preclinical safety studies during drug development. Readers are referred to other sources for a more complete list of RBC morphologies and their significance (Harvey, 2001).

#### *Heme synthesis*

The synthesis of heme involves a series of reactions that starts in the cytoplasm and mitochondria of the rubriblast, the earliest identifiable precursor of erythrocytes. Heme synthesis begins with the vitamin B<sub>6</sub>-dependent condensation of succinyl-CoA and glycine to form delta aminolevulinic acid (ALA). ALA then condenses to form

a pyrrole, and this step is inhibited by lead. Porphyrin rings are then generated and a final step incorporates an iron atom. Ferrochelatase, also inhibited by lead, catalyzes this incorporation of the ferrous iron into the tetrapyrrole protoporphyrin IX in the mitochondria (Meyer and Harvey, 1998; Kaneko, 2000).

#### *Iron metabolism*

Iron is primarily obtained from dietary sources where it is absorbed from the gut and transported as a complex with transferrin to the marrow. Iron is transferred to red blood cell precursors by receptor-mediated endocytosis. Iron stores are usually assessed by staining the marrow with Prussian blue or by measuring serum ferritin (Jain, 1993; Harvey, 1997). Cats normally lack stainable iron in their marrow (Nadrew *et al.*, 1994). Iron deficiency may result in several clinical features, such as anemia, increased erythrocyte central pallor and abnormal shapes of the red cells.

#### *Red blood cell energy requirements and hemoglobin breakdown*

The energy required by erythrocytes is especially important for the maintenance of a reduction potential to prevent hemoglobin oxidation. In mature red blood cells there are no mitochondria, therefore, the glycolytic pathway and pentose phosphate pathway are the main sources of ATP and NADPH that provide energy to prevent oxidative damage, e.g., via reduced glutathione (GSH). Aged or damaged red blood cells are removed from circulation by macrophages of the reticuloendothelial system in the spleen, liver and marrow. In the macrophage, hemoglobin is catabolized to globin and heme. The amino acids from globin re-enter the protein synthesis pool. Heme is degraded to biliverdin then to bilirubin and excreted in the urine and bile (Jain, 1993).

### **Leukocyte**

#### *Leukocyte biology*

Neutrophils primarily function in inflammatory responses and bacterial killing. With an increased demand for neutrophils, neutrophil production is up-regulated by growth factors and proinflammatory cytokines. Mature circulating neutrophils are present either in the circulating pool or the marginal pool. Neutrophils in the marginal neutrophil pool are loosely bound to capillary endothelial cells. Several physiological factors such as epinephrine and corticosteroids due to excitement and stress cause demargination of neutrophils and result in neutrophilia (Babior and Golde, 1995).

#### *Morphology of leukocytes*

Total leukocyte count refers to the total absolute count of circulating neutrophils, eosinophils, basophils,

monocytes and lymphocytes, and this number varies among different animal species. The differential leukocyte count refers to the percentage of each leukocyte population in peripheral blood and should be performed by counting and differentiating a minimum of 200 cells on a stained blood film. Granulocytes are classified according to the staining characteristics of their specific (or secondary) cytoplasmic granules. Eosinophils have pronounced acidophilic granules, basophils possess distinct basophilic granules and neutrophils have small, neutral, usually indistinct granules. The proportion of different populations of leukocytes also varies among animal species. For example, neutrophils predominate in dogs and cats, whereas they only slightly outnumber lymphocytes in horses. In ruminants and laboratory animals (rats and mice), lymphocytes predominate. The morphology of the various populations of leukocytes is fairly similar among animal species (Duncan *et al.*, 1994).

#### *Neutrophils*

Once released from the bone marrow into the peripheral blood, neutrophils circulate for 6–14h before migrating into tissues, where they survive for 1–4 days (Smith, 2000). Neutrophils account for approximately 40–70% of the total leukocyte count depending on the animal species. Mature neutrophils have a segmented nucleus with three to five lobes joined by thin strands, and they contain heterochromatic (clumped) chromatin (Figure 22.3). Occasionally, a nuclear appendage, an extra chromatin lobe resembling a drumstick, is present in neutrophils of female animals and is commonly known as the Barr body. The neutrophil cytoplasm is pale and contains a moderate number of fine pink or pale granules, depending on the animal species. Neutrophils from veterinary species are known to contain at least two types of cytoplasmic granules: azurophilic (primary) and specific (secondary) granules. In birds, reptiles and some fish, the equivalent of a neutrophil is called a heterophil, and it contains large, reddish cytoplasmic granules. The neutrophils in rabbits, guinea pigs and hamsters also contain similar-appearing granules and are therefore often referred to as heterophils. Rat and mouse granulocytes are unique in that their granulocytic precursors can develop “ring forms” in which a small “hole” is present in the center of the nucleus. These ring forms are also called “stab cells.” Ring forms are seen in neutrophil and eosinophil precursors but not in basophil precursors (Bolliger, 2004).

In healthy animals, only mature neutrophils are present in circulation. Immature neutrophils, as indicated by a decrease in nuclear segmentation, are normally restricted to the bone marrow but may be released into the blood during a granulocytic response to a disease process. Neutrophil toxicity is seen secondary to an



overwhelming demand for neutrophils, resulting in maturational defects. Neutrophil toxicity is characterized by increased cytoplasmic basophilia, foamy vacuolation and Döhle bodies (amorphous blue-gray cytoplasmic inclusions) (Figure 22.3f) (Jain, 1993; Meyer and Harvey, 1998).

### *Eosinophils*

Eosinophils are seen in low numbers, and they contain a polymorphic nucleus that is less condensed and less segmented than that of neutrophils. As eosinophils mature in the bone marrow, most primary azurophilic granules disappear or transform into specific granules. In mature cells, the pale blue cytoplasm contains mostly specific eosinophilic (red/pink) granules that are loosely packed in the cell (Figure 22.3c). The size, shape, number and staining characteristics of eosinophils vary among different animal species (Young, 2000).

### *Basophils*

Few basophils are present in normal blood. They have a segmented or irregularly shaped, often string-like, heterochromatic nucleus. The nuclear segmentation, as in eosinophils, is less pronounced in basophils. Cytoplasmic granules are metachromatic and prominent, and they stain reddish violet (Figure 22.3d). The size, number and staining reaction of granules vary among animal species. Basophils have some morphologic resemblance to mast cells, which are widely distributed in connective tissues and only rarely observed in blood. Compared to basophils, mast cells are bigger with a round nucleus and more numerous cytoplasmic granules (Scott and Stockham, 2000).

### *Monocytes*

The monocyte is generally the largest blood leukocyte and accounts for 3–8% of the total leukocyte count. Monocytes are precursors of tissue macrophages, and their nucleus can vary from bean shaped to convoluted. Their cytoplasm is abundant, blue-gray, and often appears foamy or vacuolated with a few large vacuoles (Figure 22.3e). Monocytes often have many fine or indistinct azurophilic cytoplasmic granules (Bienzle, 2000).

### *Lymphocytes*

Lymphocytes account for 20–40% of the total leukocyte count in dogs, cats and horses; 60–70% in ruminants, mice and rats; and 50–60% in pigs. Morphologically, lymphocytes are classified as small, medium and large depending on their size. The size, shape and staining characteristics of lymphocytes vary among and within animal species. Dogs and cats have mostly small circulating lymphocytes (Figure 22.3a). Both small and large lymphocytes are present in cows, sheep, goats and

rodents. In cows, large lymphocytes occasionally may have vacuolated cytoplasm and a few large azurophilic granules (Meyer and Harvey, 1998).

## **Platelet biology**

### *Platelet function*

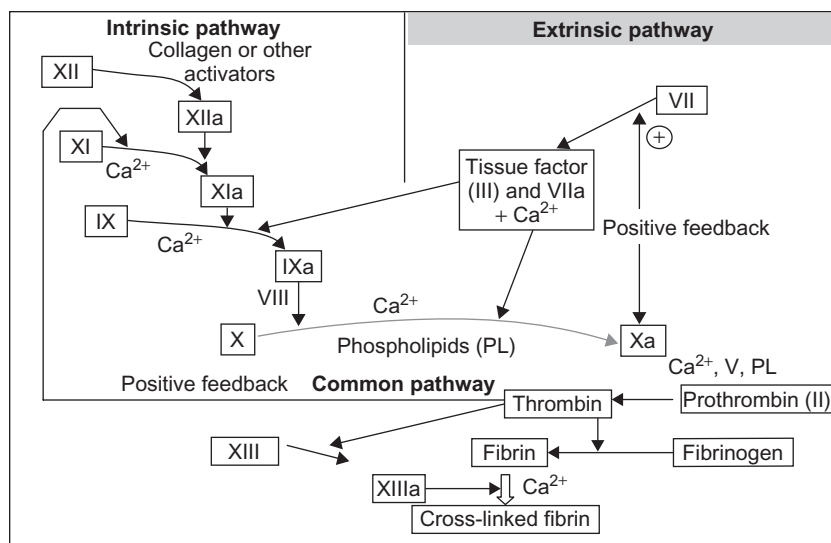
Platelets function largely in primary hemostasis. Initially, platelets adhere to the endothelium of damaged blood vessels, undergo shape changes and release proteins that cause the aggregation of more platelets to form a platelet plug and help stimulate the coagulation pathway (Figure 22.4). Platelets play a major role in secondary hemostasis as well. For example, platelets provide a surface for the assembly of several enzyme complexes that form during the process of coagulation, provide coagulation factor V and participate in the activation of factor X. Collagen stimulates platelet aggregation and platelet adhesion. Adhesion occurs between the platelet membrane and subendothelial components (collagen and fibronectin) via von Willebrand factor and glycoprotein receptors. ADP and thromboxane A<sub>2</sub> are secreted by adherent platelets and released from damaged tissue and erythrocytes, and these contribute to early platelet aggregation. Fibrinogen plays a major role in the coagulation cascade as well as in platelet aggregation (Bauer and Rosenberg, 1995).

### *Morphology of platelets*

Platelets generally vary in size and shape and are smaller than red blood cells. In stained blood films, platelets are discoid, spherical or elongated and may appear individually (Figure 22.3f) or in small to large aggregates. They are anucleate in mammals. A direct platelet count is performed on blood samples using a hematology analyzer. A subjective screening of platelet numbers (normal, increased or decreased) can also be made from their relative number on stained blood films. The terms platelet and thrombocyte are used interchangeably, although the term thrombocyte is preferentially used to describe nucleated platelets in fish, reptiles, amphibians and birds (Boudreaux, 1996; Meyer and Harvey, 1998).

### *Hemostasis*

Hemostasis occurs when there is a balance between continuous activation of the coagulation cascade and removal of the end product of coagulation, fibrin, by fibrinolysis. As mentioned previously, hemostasis can be categorized into primary hemostasis, with the formation of a platelet plug and secondary hemostasis, culminating in a fibrin clot. There are three major pathways involved in secondary hemostasis: the extrinsic, intrinsic and common pathways (Figure 22.4). In the extrinsic



**FIGURE 22.4** A diagram of the coagulation cascade consisting of intrinsic, extrinsic and common pathways. Coagulation is an enzymatic process involving the conversion of inactive enzymes to active forms resulting in amplification of the process. The final product of these pathways is the formation of cross-linked fibrin. PL is phospholipid of platelets. Roman numerals refer to the coagulation factors with these numbers. "a" following a Roman numeral indicates "active enzyme." For details, see section on platelet biology.

pathway, the initiating stimulus is the release of tissue factor from damaged endothelium and other tissues. The intrinsic pathway can be initiated by either the extrinsic pathway or contact with negatively charged surfaces such as damaged endothelium. Both the extrinsic and intrinsic pathways stimulate a series of steps, many of which are shared in the common pathway, resulting in the production of thrombin and eventually a stable fibrin clot. Calcium is a requirement in many of these steps, and phospholipids (provided by platelets) are also required for enzyme complex activity. The majority of the coagulation factors are produced by the liver. Some factors such as Factors II, VII, IX and X are dependent on vitamin K, which is obtained from the diet and produced by intestinal bacteria. Breakdown of the fibrin clot is achieved by activating plasminogen to plasmin, which degrades fibrin. Tests commonly used to measure secondary hemostasis include prothrombin time (PT), partial thromboplastin time (PTT) and activated coagulation time (ACT) (Table 22.1) (Bauer and Rosenberg, 1995; Boudreaux *et al.*, 1996).

## BIOCHEMICAL BASIS OF HEMATOPOIETIC TOXICITY

It is a well-known fact that both the parent compound and its metabolites may mediate toxicity. Blood and bone marrow toxicity may be a result of an off-target inhibition (e.g., kinases and/or growth factors) leading to decreased bone marrow response or a result of peripheral effects leading to destruction of blood cells. Alterations in the blood and bone marrow cells may also be an anticipated pharmacologic event during preclinical

drug development (Meyer *et al.*, 2010). Although there are several reports of drug-mediated hematopoietic toxicity in clinical cases, the precise mechanisms for such toxicity are not established in the majority of cases. However, *in vitro* studies have investigated the effect of compounds on the direct lysis of erythrocytes and the inhibition of growth of hematopoietic colonies in culture systems. Although mechanistically important, these results may only be extrapolated to the *in vivo* situation where the compound under test is not modified by host metabolic processes. Classic examples of a discrepancy between *in vitro* and *in vivo* studies include chloramphenicol-induced myelosuppression and 2-butoxyethanol-induced hemolysis (Turton *et al.*, 2002a, b; Corley *et al.*, 2005). The myelosuppressive activity of chloramphenicol is usually ascribed to the parent compound, but the nitroso metabolite of chloramphenicol shows a far greater inhibitory effect in mouse hematopoietic culture assays. In addition, there is considerable variation in the susceptibility of cells from different mouse strains to the nitroso metabolite. In man, nitroso-chloramphenicol has not been identified as an *in vivo* metabolite of chloramphenicol, further hampering any extrapolation from *in vitro* toxicity studies to the clinical situation (Jimenez *et al.*, 1987; Turton *et al.*, 2002b). The cleaning agent 2-butoxyethanol (2-BE) is reported to be inactive in the *in vitro* hemolysis test in rats, while oral administration in this species produces a hemolytic anemia. However, the major metabolite of 2-BE, butoxyacetic acid, produces marked swelling and lysis of erythrocytes *in vitro* (Corley *et al.*, 2005).

Another major mechanism of toxicity seen with many compounds involves the formation of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and hydroxyl radical. ROS are capable of reacting with cellular proteins, lipids and other molecules

TABLE 22.1 Commonly used tests to detect toxic effects on blood and bone marrow in clinical and preclinical toxicology

Name of test	Usefulness	Comments
<i>Complete blood count (CBC):</i> PCV, MCV, MCHC, WBC count, differential leukocyte count, platelet count	Routine blood analysis for screening effect of toxic chemicals	Modern blood analyzers report these erythrocyte indices as part of a full blood count, and manual evaluation may be necessary to confirm findings and detect unusual red and white cell abnormalities
<i>Hemolysis testing</i> (e.g., osmotic fragility test and Heinz bodies)	To confirm if hemolysis is responsible for lowered red blood cell count	Look for Heinz bodies, methemoglobinemia and eccentrocytes to detect oxidant-induced changes; spherocytes indicate immune-mediated destruction
<i>Hemostatic tests</i> (e.g., platelet numbers, PT, PTT, ACT, bleeding time)	To detect abnormalities of coagulation in the extrinsic (PT) and intrinsic (PTT, ACT) path-ways and platelet function (bleeding time)	Special tests like D-dimer assay, antithrombin, platelet function tests and individual factor assays are available in some university and commercial research laboratories
<i>Special blood tests</i> Reticulocyte count, Coombs, platelet associated immunoglobulin G (PAIgG)	These tests are based on the routine CBC findings and are considered “problem driven” tests	Reticulocyte evaluation will determine bone marrow response to anemia; Coombs and PAIgG tests may indicate an immune-mediated component in anemia and thrombocytopenia
<i>Bone marrow cytology (evaluate myeloid: erythroid ratio)</i>	Confirm blood findings and to evaluate marrow function	This is an elaborate and time-consuming procedure. Cytochemical staining can be performed to differentiate abnormal cells in cases of leukemia
<i>In vitro</i> stem cell assays using clonogenic (CFU-E, CFU-GM, CFU-GEMM) assays	Possible to examine effects on the myeloid, erythroid and megakaryocytic lineages in a fashion where concentration of the chemical and duration of exposure are tightly controlled	<i>In vitro</i> clonogenic assays have proven useful in understanding mechanisms of toxicity and in formulating strategies for treatment

leading to changes in their structure and subsequent cellular damage. Despite numerous cellular defense mechanisms such as antioxidant enzymes and low molecular weight antioxidants such as GSH, hemolysis may result if these mechanisms are overwhelmed in red blood cells. Many oxidants cause hemolysis by this mechanism (e.g., acetanilide, naphthalene, phenol; [Table 22.2](#)). One such compound that has been extensively tested is microcystin-LR, which significantly increases oxidative stress causing lipid peroxidation, changes in membrane fluidity, transformations of cell shape and hemolysis. A significant dose- and time-dependent increase in lipid peroxidation products was found in red blood cells treated with microcystin-LR when compared with untreated controls ([Sicinska et al., 2006](#)).

Non-selective kinase inhibitors can be potential bone marrow suppressants due to inhibition of key cytokines responsible for hematopoiesis. Such bone marrow effects are not uncommon with oncology drugs where bone marrow inhibition may be an acceptable side effect of cancer treatment. Ironically, chemotherapeutic and other toxic agents, particularly alkylating agents, may result in leukemia, which is likely linked to cytogenetic abnormalities, especially with the loss of part or all of a chromosome. The frequency of cytogenetic abnormalities in human patients who develop dysplastic syndrome or acute leukemia after treatment with alkylating or other antineoplastic agents ranges from 67 to 95%. However, there are other forms of leukemia that have weak correlations with exposure to alkylating agents ([Bloom and Brandt, 1997](#)).

TOXICITY-INDUCED ALTERATIONS OF  
HEMATOPOIETIC ELEMENTS

The following section discusses several mechanisms and specific causes of hematopoietic alterations. It is important to remember, however, that preanalytical factors (e.g., age, diet, fasting collection, sample handling) can have significant effects on hematological parameters and such variations should be minimized in controlled studies. A partial list of drugs/chemicals shown to have toxic effects on blood and bone marrow is included in [Table 22.2](#).

Myelosuppression

Bone marrow suppression may result from direct damage to cell precursors as well as from alterations in the cytokine profile secondary to the drug. Suppression is a widely recognized side effect of chemotherapeutic/antineoplastic agents, primarily due to the high mitotic rate of this tissue. All cell lines may be affected resulting in granulocytopenia, thrombocytopenia and/or anemia. The changes are usually dose related and reversible after withdrawal of the drug/test compound ([Turton et al., 2006](#)). The mechanisms involved in such dose-dependent myelosuppression are related to inhibitory effects on primitive cells in the marrow such as BFU-E and CFU-GM ([Deldar, 1994](#)). The inhibition of these primitive cells will manifest as erythroid hypoplasia and neutropenia, respectively,

TABLE 22.2 Few examples of drugs and chemicals associated with a variety of toxic effects and their likely mechanism of action

Drug/chemical	Effect	Mechanism of action
Chloramphenicol, sulfonamide, diclofenac, mycotoxins	Bone marrow aplasia	Unknown
Trimethoprim-sulfadiazine, cephalosporin, phenobarbital	Pancytopenia	Possibly immune-mediated destruction of stem cells
Benzene	Bone marrow aplasia	Damaged marrow microenvironment
Chlorinated hydrocarbons	Bone marrow aplasia	Stem and progenitor cell damage
Estrogen	Anemia; bone marrow suppression	Stem cell damage and decreased EPO
Arsenic	Hemolytic anemia; dyserythropoiesis	Altered DNA synthesis
Amphotericin B, insulin, isoniazid, cisplatin, rifampicin, naproxen, sulfonamide	Immune-mediated hemolytic anemia (IMHA)	Antibody-mediated destruction of erythrocytes
Aniline, acetanilide, naphthalene, phenol, nitrobenzene, sulfanilamide	Oxidative stress-induced anemia	Oxidative damage to erythrocytes
Nitrites, nitrobenzenes, phenacetin, methylene blue	Toxic methemoglobinemia	Oxidation of red blood cell hemoglobin
Warfarin, cephalosporins	Bleeding	Inhibition of the carboxylation of vitamin K-dependent coagulation factors
Heparin, gentamycin, aspirin, acetazolamide, cephalixin, gold salts	Thrombocytopenia	Immune-mediated platelet destruction

and may be due to direct cytotoxicity, inhibition or blockage of specific growth factors, or interference with some fundamental process such as DNA replication. For example, EPO production has reportedly been suppressed by excess estrogen due either to exogenous administration or endogenous release as occurs with estrogen-secreting tumors (Thrall *et al.*, 2004). Other known bone marrow suppressants whose actions are dose dependent and reversible include aminopterin, a folic acid antagonist, and benzene (Table 22.2). Azidothymidine (AZT) used in the treatment of feline immunodeficiency virus and feline leukemia virus is another example of a drug that causes anemia primarily by bone marrow suppression. Ingestion of trichloroethylene is known to cause aplastic anemia in cattle. Other chemicals/drugs known to cause bone marrow suppression include chloramphenicol, meclofenamic acid, phenylbutazone, quinidine, trimethoprim-sulfadiazine, albendazole and fenbendazole (Manyan *et al.*, 1972). Chronic treatment with recombinant human erythropoietin (rhEPO) has also been shown to cause erythroid hypoplasia and nonregenerative anemia due to the production of anti-EPO antibodies. This effect is reversible over the course of 2–12 months after discontinuation of the drug (Langston *et al.*, 2003).

## Anemia

Anemia secondary to drugs or toxins may arise due to bone marrow suppression, hemorrhage or increased destruction. A mild anemia is a common feature in pre-clinical toxicology studies in which high doses of a drug or a chemical are administered. The anemia is usually

secondary to toxicity affecting other organ systems and is thought to be similar to anemia of inflammatory disease often seen in clinical cases. This finding is usually nonspecific and insignificant. Another common reason for mild anemia in toxicology studies is attributed to the combination of frequent blood sampling for toxicokinetic studies and the stress of repeated handling, especially in primates (Hall, 1992; Gossett, 2000). In the bone marrow, decreased red blood cell precursors can also be noted in toxicology studies concurrent to decreased food intake and loss of body weight (Reagan *et al.*, 2011). If prolonged, this could lead to anemia.

### Immune-mediated hemolytic anemia

Drug-induced immune-mediated hemolytic anemia (IMHA) in humans is thought to occur as a result of the following three mechanisms: formation of antibodies (IgG, IgM or IgA) to drug adhered to red cell membranes (hapten or neoantigen formation), binding of preformed drug-antibody complexes to the red cell membrane, or autoantibody formation. However, documented cases of drug-induced IMHA in animals are infrequent and most are probably related to hapten formation. Examples of IMHA caused by drugs in animals include penicillins and cephalosporins, which are known to cause anemia by a combination of immune-mediated hemolysis and bone marrow suppression. Levamisole is another drug suspected of inducing IMHA in dogs that are being treated for heartworm disease. Vaccination has also been implicated in cases that present with IMHA within short periods of time after vaccination. However, the precise mechanism for such a response due to vaccination is not known at present (Bloom *et al.*, 1988; Andrews, 1998; Gossett, 2000).



### *Oxidative or nonimmune hemolysis*

Exposure to oxidants results in the production of methemoglobin and/or large aggregates of oxidized, precipitated hemoglobin that are attached to the internal surface of erythrocyte membranes (Heinz bodies, Figure 22.3b). Eccentrocytes are also often formed with oxidative damage resulting from altered cytoplasmic-membrane adhesions. The attachment of Heinz bodies to the plasma membrane causes membrane rigidity with subsequent red blood cell lysis and anemia. Several oxidants are implicated in oxidative hemolysis caused by Heinz bodies. Examples in dogs and/or cats include methylene blue, onions, acetaminophen, phenazopyridine and propylene glycol, and phenothiazine in horses. Zinc-induced hemolytic anemia may occur due to ingestion of metallic hardware items, pennies and topical formulations containing zinc oxide. Sudden release of copper from accumulations in the liver of several species (particularly sheep) has been reported to cause Heinz bodies and an acute hemolytic crisis. Vitamin K and DL-methionine induce methemoglobinemia in multiple species, although Heinz bodies and anemia are not frequent (Houston and Myers, 1993; Schlesinger, 1995).

### *Basophilic stippling*

Basophilic stippling is usually seen in mature erythrocytes and is thought to be due to the presence of aggregates of ribosomes and polyribosomes. Basophilic stippling usually represents a regenerative response to anemia in ruminants and less commonly in other species. It may also be seen in lead poisoning, likely due to retention of RNA (Weiss, 2010). Siderotic inclusions (Pappenheimer bodies) in the red blood cells resemble basophilic stippling. Siderotic inclusions contain iron and may be seen after isoniazid therapy, lead toxicity, chloramphenicol and zinc toxicity.

### *Neutrophilia and neutropenia*

An elevated neutrophil count is often present in cases of bacterial infection and many inflammatory conditions, although initially a neutropenia may be detected. In addition, chemicals such as lead, mercury, phenacetin and pyridine may induce neutrophilia as a secondary response to tissue damage or hypersensitivity reactions. Neutrophilia is often seen in laboratory animals as a secondary event in neoplastic conditions or following hemorrhage. Increased neutrophil production and delivery to the circulation in response to inflammation may be the result of multiple signals including endotoxin, interleukins, interferons and cytokines (Anderson, 1993). Approximately 3–5 days are required for a significant increase in neutrophils to be observed peripherally due to increased bone marrow production (Duncan *et al.*,

1994). Neutropenias may be observed due to destruction of mature neutrophils, myeloid precursors or supporting cells. Neutropenia may also be secondary to attenuation of inflammation by a drug rather than suppression of granulopoiesis, as seen with a selective JAK inhibitor (Meyer *et al.*, 2010).

### *Eosinophilia, monocytosis and lymphocytosis*

Eosinophilia is seen in parasitic infections, dermatitis and occasionally in allergic reactions to drugs such as salicylates. Increased eosinophils may be seen in the peripheral blood and wall of the rat gut following the use of dietary expanders such as modified starches and sugars (sorbital, mannitol, lactose and polyethylene glycol). Eosinophilia has also been reported with excessive fluoride ingestion in rats (Andrews, 1998).

Monocytosis is often seen concomitant with neutrophilia and is usually a nonspecific finding of inflammation or stress. It is also seen acutely after trauma such as accidental gavage of the lung in rodents. Monocytosis has been described resulting from the administration of the antipsychotic drug chlorpromazine (Andrews, 1998).

Increased lymphocyte numbers may be present peripherally following immunization and with physiological excitement due to epinephrine (Meyer and Harvey, 1998).

### *Erythrocytosis and thrombocytosis*

Increased numbers of erythrocytes (erythrocytosis) seen in peripheral blood is usually a relative versus absolute increase, meaning that it usually does not represent a true increase in red cell mass. It is frequently associated with hemoconcentration/dehydration due to decreased food intake or fluid loss such as diarrhea and vomiting. The increased erythrocyte counts in these situations may therefore only reflect the change in the plasma compartment rather than alterations in the production or fate of red blood cells. A sudden release of erythrocytes due to splenic contraction is another cause of a relative erythrocytosis (Duncan *et al.*, 1994). In pre-clinical studies, a pharmacologically absolute erythrocytosis may be expected if either EPO activity or enhancement of EPO activity is present.

Increased platelets may be seen with blood loss, inflammation and iron deficiency. The cytotoxic drug 5-fluorouracil causes an immediate fall and then a sustained increase in platelet numbers. Other compounds causing thrombocytosis are the drugs AZT and vincristine and the food additive butylated hydroxytoluene. The mechanisms involved in drug-related thrombocytosis are unclear, although cytokines including stem cell factor and interleukin-6 have been implicated. Thrombocytosis is often synchronous with

reticulocytosis, supporting the hypothesis that EPO has a stimulatory effect on megakaryocytes (Boudreaux *et al.*, 1996).

#### *Thrombocytopenia/blood loss/coagulation inhibition*

Thrombocytopenia may be an artifactual finding due to platelet aggregation during or after sample collection, and this must first be ruled out as a cause before additional investigation is performed. Thrombocytopenia caused by drugs and chemicals may be directly caused by their cytotoxic effect on bone marrow progenitor cells, as noted with chemotherapeutic agents, by peripheral destruction due to immune-mediated mechanisms or by increased consumption due to a coagulopathy. Other than chemotherapeutic agents, chloramphenicol has been associated with thrombocytopenia in dogs and cats. Similarly, estrogen and interferons can cause destruction of stem cells resulting in marrow suppression and thrombocytopenia. Agents that can specifically destroy megakaryocytes include anagrelide, ethanol, estrogens, interferons and thiazide diuretics (Anthony *et al.*, 1994; Okamura *et al.*, 1994; Rodman *et al.*, 1997). Drugs associated with peripheral destruction of platelets include methyl dopa, levodopa and gold therapy. Toxins that induce thrombocytopenia include aflatoxin B1, brackenfern and trichloroethylene (Weiss, 2000). Xenobiotic-induced platelet destruction is due to either immune-mediated or nonimmunologic destruction (Bloom *et al.*, 1988; Shebuski, 1993). Hapten (neoantigen/compound epitope formation), induction of antibodies and immune complex formation are all possible mechanisms of immune-mediated platelet destruction.

The two main toxicologic consequences of platelet modulation are predisposition to hemorrhage and thrombosis. Nonsteroidal anti-inflammatory drugs have been known to cause blood loss anemia, which is thought to be due to gastrointestinal damage or ulceration secondary to the local inhibition of prostaglandin synthesis (George and Shattil, 1991). Aspirin decreases platelet function and aggregation and can also cause gastrointestinal ulceration resulting in hematemesis and melena in humans and animals.

Coumarin-like compounds, such as warfarin, inhibit the carboxylation of vitamin K-dependent coagulation factors II, VII, IX and X resulting in prolonged clotting times (Bloom, 1997; Bloom and Brandt, 1997). Cephalosporins are also responsible for prolonged clotting times by the same mechanism. Several antibiotics that cause eradication of gut microflora and thus lower the levels of available vitamin K can prolong coagulation and cause bleeding. Any toxic compound that results in liver failure (such as acetaminophen) will cause decreased synthesis of the coagulation factors needed for clotting and may result in bleeding.

#### *Toxic leukemogenesis*

A few examples of leukemogenic agents include aromatic hydrocarbons (benzene), alkylating chemotherapeutic agents and ionizing radiation. The development of leukemia is not highly predictable or dose related in humans (Irons, 1997). Exposure to chemical clastogens that cause chromosomal damage (benzene and its metabolites) results in genetic damage. In many cases the damage is reversible by DNA repair mechanisms. For example, the folic acid antagonist methotrexate causes transient *in vitro* DNA damage, but therapeutic use of the drug is not associated with the development of leukemia. However, a secondary insult may overcome natural DNA repair capability resulting in permanent chromosomal damage. Acute myeloid leukemia can be induced in rats by very small quantities of dimethylbenzanthracene or with ionizing radiation (Levine and Bloomfield, 1992; Andrews, 1998).

## CONCLUSIONS AND FUTURE DIRECTIONS

The hematopoietic system is very susceptible to a variety of toxic chemicals and drugs, and such toxic effects may result in financial liability in cases of preclinical drug development and mortality in clinical practice. Because of this, systematic examination of blood and bone marrow is a major requirement of clinical and preclinical toxicity studies, and it has become a routine part of diagnostic evaluation in the clinics when screening for toxic effects of drugs. In most cases, bone marrow toxicity can be assessed by evaluating complete blood cell counts, peripheral blood smears and bone marrow examination. Finding toxic effects of a test compound in a dose-related fashion and without adequate therapeutic margins can typically halt further development of the drug.

Compared to other areas of toxicology, the blood and bone marrow effects noted in preclinical species is often reliably translated to the clinics, since the pathologic mechanisms responsible for such effects are mostly similar across species (Reagan *et al.*, 2011). In addition, if an adverse finding is demonstrated preclinically but an adequate therapeutic index is present, a routine blood smear examination can easily be performed to monitor adverse findings in clinical patients.

In conclusion, it is possible for a toxicologist to systematically evaluate blood and bone marrow toxicity in laboratory settings and to devise a hypothesis for development of such toxicity. Because of this, it is imperative for toxicologists to understand the basic concepts of the hematopoietic system in order to interpret the results of toxicological studies.

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# Immunotoxicity

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## INTRODUCTION

The immune system has developed to counter hazardous molecules and biological factors that are foreign to the host. Unlike invertebrates, humans and vertebrate animals have the ability to develop an immune response that retains memory for the invading agent. Immune response may manifest as specific antibodies, proliferation of specific cytotoxic cells and clonal expansion of memory cells toward the invading agent. Generally, an immune response is protective and results in inactivation or destruction of the potentially harmful chemical or a pathogen. Because of its protective effects, the immune system provides strong survival advantage. Immunotoxicology is the science that evaluates the undesirable effects of chemicals and other toxic agents on the host immune system. Although intuitively the effects of water and air pollutants and food additives would be highly relevant to the health of domesticated and farm animals, veterinary immunotoxicology has not been pursued to any significant extent (Kende *et al.*, 1984). Therefore, the immunotoxic inferences described in this chapter are derived primarily from human epidemiology and animal models. Many of the health effects of chemicals, including those commonly used in the agricultural industry such as herbicides and pesticides, might be contributed by their immunotoxic effects. Pathogens such as tuberculosis and human immunodeficiency virus (HIV) promote their survival through their ability to compromise the immune system.

## Historical background

Although the modern discipline of immunotoxicology is relatively new and immunology itself is nearly a century

old, concern with infections and their possible prevention dates back nearly one thousand years. It was realized long ago that survivors of smallpox or plagues were relatively resistant to the disease they had acquired earlier, and people in China, for example, were inhaling dried smallpox scabs to prevent infection as early as the 11th century. It was not until the late 19th century that boosting immunity with vaccination for specific infections was possible. The cellular basis of immune responses was recognized in the middle of the 20th century. The same period also saw the realization that inadvertent exposure to chemicals and even the therapeutic use of drugs may influence the optimal mounting of immune responses against invading organisms. There were occasional published reports of immunotoxic effects by environmental chemicals, especially pesticides, prior to 1970; a systematic study of immunotoxicity of various chemicals followed that period.

The search for drugs that modulate immune functions predates the experimental approach in immunotoxicity and has also provided valuable information about immune mechanisms. It was also known some time ago that environmental factors are important for development of cancer or certain autoimmune diseases; this information has led to screening of various chemicals for their untoward effects on the immune system. Yet, the information regarding the immunotoxicity of chemicals is often incomplete, controversial and has little relevance to doses or concentrations which are encountered in normal environmental exposures.

## Immunotoxicity in animal health

The study of immunotoxicity in a veterinary setting is an important issue. Although the study of veterinary immunotoxicology has not been pursued extensively, its

relevance to animal health is obvious. Most of our information on veterinary immunotoxicity comes from the extension of results from rodent experimental models. Some of the relevant areas are discussed below.

### *Infectious diseases*

The immune response is critical to the survival of the host against infections. Following an infection, a vertebrate defends itself by relatively non-specific innate and specific adaptive immune responses. Even a mild infection in an immunosuppressed host may cause life-threatening illness or death. Children and young animals are not fully immunocompetent, and weaker immunity may suppress growth and productivity in livestock. For example, because of relative weak immunity, waterborne *Cryptosporidium* may cause cryptosporidiosis – an opportunistic infection in immunocompromised animals, leading to significant economic losses.

### *Interference with vaccination*

Many chemical contaminants in water and soil have been shown to be immunosuppressive in animal models. For example, in the U.S., livestock is routinely vaccinated against common epizootic infections and the health of the vaccinated animals has notably improved. However, the efficacy of these vaccines may be compromised by immunosuppressive chemicals in the environment. Therefore, understanding the immunotoxicity of chemical contaminants at environmentally relevant concentrations is very important to ascertain their effects on animal health and efficacy of vaccines.

### *Immunotoxicity and cancer*

Environmental chemicals have the potential to cause cancers. This has been shown to be the case in experimental animal models with chemicals such as polycyclic aromatic hydrocarbons. Both innate and adaptive immune responses play a role in cancer surveillance, but immunosuppression induced by environmental chemicals may induce and promote tumor growth. Athymic mice lacking functional T cells are more susceptible to transplanted tumors suggesting that T cells are important in limiting the growth of tumors. Moreover, activation of cancer-specific immunocompetent cells is a promising new method for cancer treatment (Quezada *et al.*, 2011). Thus, exposures to immunosuppressive chemicals may lead to increased incidence of tumors and higher morbidity and mortality in livestock.

### *Autoimmunity*

An immune response is an exquisitely well-balanced act with built-in positive and negative modifiers. While weaker immune responses may cause immunodeficiency

and increased susceptibility to diseases, excessive immune response may lead to autoimmune disorders. Many small molecules are not immunogenic *per se*, but may interact with other molecules such as tissue proteins, leading to an immune response to “modified self” antigens and development of autoimmunity and hypersensitivity reactions. This has been elegantly demonstrated with many different small molecules, including procainamide, iodine and dioxin (Pollard *et al.*, 2010). Similarly, some infections may promote immune responses to self antigens through cross-reactivity. Thus, human rheumatoid heart disease may develop from streptococcal infection (Guilherme and Kalil, 2010). A number of autoimmune disorders in humans and animals are controlled by genetic susceptibility, but environmental chemicals may be a trigger in genetically susceptible individuals. In humans, autoimmune disorders are generally treated with immunosuppressive drugs; however, this option may not be economically practical in livestock.

## IMMUNE SYSTEM

To understand immunotoxicology, it is important to summarize basic concepts of immunology. A detailed description of the immune system can be found in *The Immune Response, Basic and Clinical Principles* by Mak and Saunders (2006). Vertebrates are capable of two types of immune responses: innate and adaptive (acquired) immunities. Although both innate and adaptive immune responses distinguish between self and non-self antigens, the mechanisms involved in the recognition of self and non-self antigens are different between the two responses. Both types of immune responses must operate normally to achieve optimal defense against pathogens. Generally a foreign entity is recognized by one or more subtypes of immune cells at mucous membrane interfaces – potential entry sites for pathogens, leading to initiation of an immune response.

### Cellular components of the immune system

The immune system comprises a wide variety of cells that communicate with each other and other cell types to mount an effective immune response. Different types of immune cells and their surface markers are listed in Table 23.1. Cells mediating innate and adaptive immune responses are white blood cells or leukocytes. In mammals the primary source of leukocytes is bone marrow and the thymus, and there are several different subtypes of leukocytes. These cells are found throughout the body and are described on the following page.

TABLE 23.1 Cellular components of the immune system and their functions in mammals

Cell types	Specific cell surface markers	Functions
Stem/progenitor cells	CD34, CD90, CD110, CD111, CD117, CD133, CD202, CD243	Differentiate into hematopoietic cells, including erythrocytes and leukocytes
T lymphocytes	CD3, CD4, CD5, CD7, CD8, CD25, CD28, CD45R, CD52L, CD69, CD134, CD152, CD154	Generation of Th and cytotoxic T cell subsets, memory T cells and production of cytokines
T helper lymphocytes (Th1, Th2 and Th17)	CD2, CD3, CD4, CD28, CD45R, IL23R	Help maturation of T and B cells
Cytotoxic T lymphocytes (CTLs)	CD3, CD8, other lymphocytic markers, but not CD4 and IL-23R	Cause lysis of specific antigen-bearing target cells; graft rejection
NKT cells	CD11b, CD16a, CD56, CD69, CD94, CD152, CD158a, CD161	Lysis of viral-containing cells and tumor cells
B cells	Lymphocyte markers + CD21, CD35, CD40, CD45	Differentiation into antibody producing cells, antigen presentation to T cells
Plasma cells	CD38	Antibody production
Monocytes, macrophages	CD11b, CD13, CD14, CD64, CD80, CD86, CD115, MHC class II	Phagocytosis, inflammatory responses, cytokine production, antigen presentation to T cells
Dendritic cells	CD1a, CD11c, CD80, CD83, CD86, CD123, CD205, CD207, CD209	Antigen presentation to T cells
Mast cells	CD33, CD117, CD203c	Release of vasoactive products, anaphylaxis, wound healing, defense against pathogens
Granulocytes	CD15, CD16b, CD31, CD88, CD156a	Phagocytosis, inflammation

Phenotypes indicated refer to human cells; only human and mouse cells are extensively analyzed. Only differentiating markers are indicated. Information on most domestic animals is limited.

Source: eBioscience, San Diego, CA.

### Neutrophils

Neutrophils, also referred as polymorphonuclear neutrophils (PMNs), are granulocytes. They are the most abundant type of leukocytes in mammals and contain a nucleus divided into 2–5 lobes. While they are normally found in the blood, they are the first responders during acute inflammation as a result of infection or tissue injury. Chemotaxis allows them to migrate to the site of inflammation/injury where they release cytokines that amplify the inflammatory response. Neutrophils provide the front-line defense against invading pathogens by digesting them in phagosomes (Segal, 2005). Neutrophils act by ingestion (phagocytosis), release of anti-microbial granules and formation of “extracellular traps” composed of chromatin fibers and serine proteases (Brinkmann *et al.*, 2004). Low neutrophil count (neutropenia) makes the host highly susceptible to infection.

### Macrophages

Macrophages are differentiated monocytes in tissues. Like neutrophils, macrophages are phagocytes and can engulf pathogens and cell debris, removing necrotic cells from the site of inflammation, including neutrophil debris. In mice, macrophages express CD14, CD11b and F4/80. Macrophages are important in both innate and adaptive immunities. Trapped material such as pathogens is taken into phagolysosomes where enzymes and toxic peroxides digest the pathogens and other trapped cellular proteins.

In addition to innate immunity, macrophages have the ability to present processed antigens to T cells to initiate the adaptive immune response. They may also help in muscle regeneration by elaborating soluble substances that stimulate proliferation, differentiation, repair and regeneration of muscle. Macrophages have different names in different tissues: dust cells/alveolar macrophages in the lung, histiocytes in connective tissue, Kupffer cells in the liver, microglia in the nervous tissues, epithelioid cells in granulomas, osteoclasts in the bone and sinusoidal lining cells in the spleen. Some bacteria and viruses are resistant to lysosomal degradation in macrophages and these cells become sanctuaries for these pathogens, including *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV).

### Dendritic cells

Dendritic cells have dendrites (branched projections) and act as highly potent antigen-presenting cells (Banchereau and Steinman, 1998). Dendritic cells are present in tissues that are in close contact with the environment, including the skin (Langerhans cells) and inner lining of the nose, the lung, the stomach and the intestines. Once activated the cells migrate to lymph nodes, where they interact with T cells and B cells to initiate the adaptive immune response (Sallusto and Lanzavecchia, 2002). Dendritic cells are of two types: myeloid and plasmacytoid. Myeloid dendritic cells evolve from myeloid

precursors, express Toll-like receptor 2 (TLR2) and TLR4, and produce IL-12 upon activation. Plasmacytoid dendritic cells are derived from lymphoid precursors, express TLR7 and TLR9, and secrete large amounts of type I interferon (interferon- $\alpha$ ).

### Natural killer (NK) cells

NK cells are large granular lymphocytes that possess the ability to kill targets without specific antigenic activation. These cells play a major role in the surveillance of spontaneous tumors and death of virus-infected cells. Several cytokines have been implicated in the activation of NK cells; these include IL-12, IL-15, IL-18, IL-2 and CCL5. NK cells also express CD16, that upon binding to the Fc portion of an antibody, kills the cells through antibody-dependent cellular cytotoxicity (ADCC). Cytoplasmic granules in NK cells contain perforin and granzyme that cause pore formation and lysis, respectively, in target cells (Oldham, 1983).

### B cells and T cells

These are two major lymphocyte subtypes that are involved in adaptive immunity. Both T and B cells are derived from bone marrow cells; however, while B cells mature in the bone marrow, T cell maturation occurs in the thymus. In the thymus, through positive and negative selections, T cells learn to distinguish between “self” and “non-self,” which is critical for the generation of tolerance to self antigens (Klein *et al.*, 2009). T cell and B cell receptors are clonally generated by random recombination of certain segments of DNA. T cell receptors (TCRs) and B cell receptors (BCRs) have enormous diversity and each naïve cell recognizes a unique antigenic determinant (epitope). B cells produce and secrete antibodies, while T cells differentiate into helper and cytotoxic T cell types. B cells can bind soluble antigens, but T cells recognize antigenic determinants only in association with self major histocompatibility (MHC) antigens on an antigen presenting cell. Depending on the type of TCR expressed, there are two major classes of T cells:  $\alpha/\beta$  TCR-containing cells (>90%) express the TCR that is a heterodimer of  $\alpha$  and  $\beta$ , while  $\gamma/\delta$  T cells (<10%) express TCR made from a heterodimer of  $\gamma$  and  $\delta$  TCR chains; compared to  $\alpha\beta$  TCR,  $\gamma\delta$  TCR exhibits limited diversity. Mature T cells with  $\alpha\beta$  TCR have two major subtypes:  $CD4^+$  and  $CD8^+$ .  $CD8^+$  T cells bind epitopes that are part of class I MHC antigens and  $CD4^+$  T cells recognize antigenic epitopes in association with class II MHC antigens. Both B and T cells have the ability to become memory cells that recognize specific antigens.

T-helper (Th) cells are  $CD4^+$  T cells which can be functionally divided into three subtypes: Th1 cells primarily produce interferon- $\gamma$  and proliferate in response to IL-12. They act as helper T cells for generation of cytotoxic T cells. Their function is regulated by STAT1 and STAT4,

and they contain the lineage-specific transcription regulator T-bet. Th2 cells are the major producers of IL-4, IL-5 and IL-13, and proliferate in response to IL-4. Th2 cells play a critical role in the defense against parasites and are also involved in allergic responses and asthma. The function of Th2 is regulated by STAT6 and contains the lineage-specific transcription factor GATA3. Th17 has been recently recognized as a new lineage of  $CD4^+$  T cells that express IL-23 receptor and produce IL-17 and IL-21. Th17 cells proliferate in response to IL-6 and IL-13, and contain the lineage-specific transcription factor ROR $_{\gamma t}$ . They are proinflammatory and play an important role in the host defense against infection by recruiting neutrophils and macrophages to infected tissues. Th17 cells are increasingly being recognized as key players in autoimmune and inflammatory diseases (Dong, 2008). Table 23.2 describes major cytokines and their functions.

### General features of innate immunity

The immune system is primarily built to defend against infections that result from attachment and entry of pathogens into the body. Apart from acquired immunity, the body has several defense mechanisms to thwart and limit infections. Skin is the largest organ in the body and forms a strong physiological barrier against the entry of pathogens into the body. Secretions such as mucus in the airways (mucociliary train) and gut, and acid and hydrolyzing enzymes in the stomach, help to clear the invading pathogens before they make contact with tissues.

The innate immune response does not make fine distinctions among invading entities and does not cause permanent changes in the immune system (i.e., the response to a second exposure by the same foreign entity does not differ in the kinetics or the magnitude from the response to first encounter).

Specialized phagocytic cells such as neutrophils and macrophages express cell surface receptors to recognize pathogens and their molecules. In recent years, TLRs have been intimately linked to innate immunity and shown to be critical in the recognition of microbial elements and their products. TLRs are a group of pattern recognition receptors (PRRs) that recognize microbe-associated molecular patterns (MAMPs) such as lipopolysaccharides, bacterial proteins, CpG DNA motif, double stranded RNA and flagellin. Table 23.3 shows specific ligands (MAMPs) of some important TLRs.

TLRs are type I transmembrane proteins composed of extracellular, transmembrane and cytoplasmic domains. The extracellular domain recognizes specific microbial elements and the intracellular domain mediates the intracellular signaling as a consequence of interaction between TLRs and MAMPs (Palm and Medzhitov, 2009). So far ten TLRs have been identified in human genome (TLR1-TLR10). TLR1, TLR2, TLR4, TLR5 and TLR6 are anchored



TABLE 23.2 Important cytokines and their major sources of production

Cytokine	Source(s)	Function
TNF $\alpha$	Macrophages, mast cells, NK cells	Regulation of cytokine expression and cell adhesion molecules; tumor cell death
TNF $\beta$	Th1 and cytotoxic T cells	Phagocytosis, cell death; NO production
IFN- $\gamma$	Macrophages, Th2 cells, NK cells, activated B cells	Elimination of pathogens; switching of Ig classes; inhibits viral replication and cell proliferation; homeostasis of respiratory epithelium
TGF $\beta$	Monocytes, T cells	Chemotaxis of macrophages; IL-1 activation; IgA synthesis; differentiation of Th17 cells
MIF	Macrophages, lymphocytes	Chemotaxis of monocytes and T cells
IL-1 $\alpha$ and IL-1 $\beta$	Monocytes, macrophages, dendritic cells; B cells	Activation of Th and NK cells; B cell amplification; inflammation
IL-1Ra	Macrophages, monocytes	Inhibits IL-1 and IL-2 action
IL-2	Th1 cells	Activation, proliferation, differentiation of T cells, B cells and NK cells
IL-3	Th cells, NK cells, monocytes	Growth and differentiation of stem cells and mast cells; degranulation of mast cells
IL-4	Th2 cells	T cell and B cell proliferation and differentiation; MHC II expression on macrophages
IL-5	Th2 cells	Proliferation and differentiation of activated B cells; IgA synthesis; eosinophil growth
IL-6	Macrophages, monocytes, Th2 cells, stromal cells	Maturation of B cells into plasma cells; antibody secretion; differentiation of stem cells; acute phase response; inflammation; inhibition of Tregs
IL-7	Bone marrow and thymus stromal cells	Differentiates stem cells into progenitor T and B cells
IL-8	Macrophages, endothelial cells	Neutrophil chemotaxis
IL-10	Tregs	B cell activation; inhibition of Th1 responses
IL-12 (p70 and p40)	B cells, monocytes, dendritic cells	Differentiation and activation of cytotoxic T cells and NK cells; induction of IFN
IL-13	Th2 cells	Th2 cell differentiation; mucus production in the lung; B cell proliferation; defense against nematodes
IL-15	Dendritic cells, macrophages, epithelial cells	NK cell development; $\gamma\delta$ T cell development; mast cell proliferation
IL-17	Th17 cells	Inflammatory response and defense against pathogens; autoimmune diseases
IL-21	Th17, NKT cells	Differentiation of Th17 cells; inhibition of Tregs; autoimmune diseases
IL-23	Macrophages, dendritic cells	Proliferation of Th17 cells; autoimmunity

TABLE 23.3 Ligands for some Toll-like receptors

TLRs	MAMPs
TLR2	Bacterial lipoproteins, lipoteichoic acid, peptidoglycan, Zymosan
TLR3	Double stranded RNA
TLR4	Lipopolysaccharide
TLR5	Flagellin
TLR & TLR8	Single stranded RNA
TLR9	Unmethylated CpG DNA

into plasma membrane, while TLR3, TLR7, TLR8 and TLR9 are endosomal. Interaction between TLRs and its ligands leads to activation of mitogen-activated protein kinases that leads to the activation of transcription factors such as the nuclear factor  $\kappa$ B (NF- $\kappa$ B), activator protein-1

(AP-1) and interferon-regulatory factors (IRFs); these factors are involved in immune and inflammatory responses against microbes. Impaired microbial recognition through genetic defects in PRRs may lead to serious diseases. For example, aberrant TLR5 activation has been linked to inflammatory bowel disease in human and rodents. TLRs also promote acquired immunity through induction of costimulatory molecules on antigen-presenting cells (Li *et al.*, 2009). Thus, TLRs are important both for innate and adaptive immune responses.

### General features of adaptive immunity

Adaptive immunity differs from innate immunity with respect to specificity, memory, diversity, tolerance and “division of labor” (Mak and Saunders, 2006).

### Specificity

Specificity indicates that the response is specific to a unique antigen either by the antibody produced by B cells or effector T cells expressing antigen-specific receptors. T and B cells expressing randomly generated TCR and BCR, respectively, are selected by and proliferate in response to specific antigenic epitopes. However, antigens with closely related epitopes to the inducing antigen may exhibit some degree of cross-reactivity with the effector cells or the antibodies to the antigen. Nonetheless, unlike the broad recognition of TLRs, the range of binding specificities of a lymphocyte is very limited.

### Immunological memory

An adaptive immune response is characterized by development of memory that facilitates the response to the repeat antigen both in time and magnitude. This immunological memory is the basis of vaccination and development of secondary immune response that protects the host from the same or similar pathogen. The mechanism of memory cell development is not fully understood; however, during the antigen-induced clonal expansion of lymphocytes, some daughter cells differentiate into long-lived memory cells that bear the antigenic receptors identical to the TCR involved in initial recognition of the immunizing epitope.

### Diversity

An innate immune response has a limited capacity to recognize an antigen. On the other hand, the capacity of the adaptive immune response is limitless with an estimated number of distinct randomly generated receptors that recognize antigens exceeding  $10^{12}$ . This incredible potential number of receptors suggests that mammals are capable of responding to any conceivable molecule. As discussed under immunological cells, this degree of diversity in BCRs and TCRs arises through somatic gene rearrangement during the development of B and T cells.

### Immunological tolerance

Because we have BCRs and TCRs that recognize virtually anything, there has to be a mechanism that limits our ability to react to our own (self) antigens. Tolerance to self antigens results from the screen in the thymus through the positive and negative selection processes that eliminate T lymphocytes with significant self reactivity. Similarly, maturing B cells undergo a central tolerance process in the bone marrow. In addition, there are peripheral tolerance mechanisms that operate outside the thymus and bone marrow and ensure that a normal host does not elicit significant immunological reactivity to self antigens. Discussion of the peripheral tolerance mechanisms is beyond the scope of this chapter and

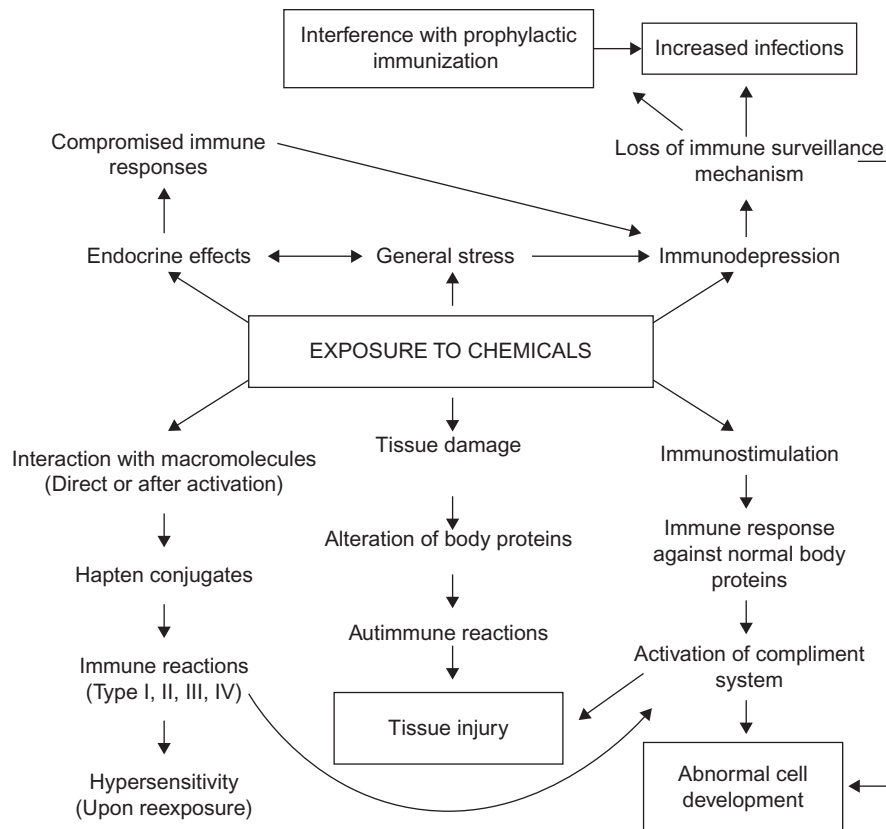
is reviewed in Yarkoni *et al.* (2010), Thomas (2010) and Mueller (2010). Breakdown of immunological tolerance may lead to autoimmune diseases.

### Division of labor

Pathogens come in different flavors; some are intracellular while the others are extracellular. Similarly, immune responses against cancer cells and transplanted tissues would be somewhat different from those against pathogens. The adaptive immune response has evolved to entrust different jobs to different effector cells. Thus, B cells recognize extracellular bacteria or bacterial products and mount the humoral (antibody) response. Binding to the antibody marks these molecules/cells for elimination by phagocytic cells such as macrophages. T cells are incapable of recognizing soluble antigens; therefore, the B cell is the primary cell type to defend against extracellular entities. On the other hand, antibodies are unable to penetrate cell membranes to attack intracellular pathogens. This is accomplished by the cell-mediated immune response that is mounted by T cells. T cells recognize foreign antigens on the cell surface of an infected cell and generate the antigen-specific cytotoxic T cells that kill the host cells carrying the foreign antigen. Both cytotoxic T cells and B cells require products of T helper cells for proliferation and differentiation. Helper T cells have the function to help the growth and differentiation of B cells and cytotoxic T cells. T cells such as regulatory T cells (Tregs) help in containing the innate and adaptive immune responses from going into overdrive and causing tissue damage.

### Relationship between innate and adaptive immune responses

The innate immune response is critical in limiting the access of pathogens until the development of specific lymphocytes of the adaptive immune response. Therefore, the innate immune response is the first line of defense against pathogens. It should be emphasized that in many instances the innate immune response successfully eliminates pathogens even before the adaptive immune response becomes functional. Moreover, the molecules essential for development of the adaptive immune response are produced by the cells of the innate immune system. In addition, the cells in the innate immune response synthesize chemokines (chemo-attractants) that guide the migration of the effector lymphocytes to the site of infection/injury. Similarly, the activated lymphocytes secrete products that activate large numbers of the innate immune response cells. Thus, the two responses are interdependent for optimal defense against pathogens.



**FIGURE 23.1** A hypothetical scheme depicting various pathways by which a chemical may produce immunotoxic effects, immune-mediated tissue injury and/or disease. Adapted from [Sharma \(1984\)](#).

### Possible mechanisms of immunotoxicity

Exposure of animals to toxicants may have effects on their immune system by a variety of mechanisms. Not only is the immune system profoundly influenced by other organs or systems of the body, but the complexity of immune responses also provides multiple targets for effects of chemicals. A generalized scheme of how chemicals may influence the immune system is illustrated in [Figure 23.1](#). Both immunosuppression and excessive immunostimulation are undesirable for the general health of animals. Chemicals may affect protein synthesis required for cell proliferation as well as antibodies. Chemicals that have potential for alkylating nucleic acids or proteins are immunotoxic because they interfere with the function of various proteins in the immune responses. Some mycotoxins or their metabolites are highly reactive in mammals and may bind to or destroy tissues. The acquired immunity depends largely on cellular proliferation and any substance that interferes with proliferation of immune cells will cause immunosuppression.

Chemicals may interfere with the function of enzymes directly, which is required in the immune processes.

Activated esterases may degranulate mast cells, releasing histamine, and may be involved in the process of chemotaxis by polymorphonuclear cells. Protease inhibitors will interrupt the post-translational processing of various cytokines and can interrupt their release. Chemicals likely to modify cell membrane functions will have a similar effect. By altering cytokine signaling, chemicals may profoundly alter differentiation of immune cells, such as lymphocyte subtypes, and since various cellular components have synergistic or antagonistic function, the homeostasis of the immune system is likely to be disrupted. Some chemicals, particularly biologically reactive immunogens, can selectively eliminate specific cells of this system. For example, chemicals that can destroy macrophages will interrupt antigen processing and presentation to other cells for mounting an effective immune response.

Immune responses may cause tissue damage or can be mediators for causing health problems in animals. Allergy or hypersensitivity is a good example where the well-being of the animal can be compromised. The allergic reactions require initial sensitization and later a challenge exposure to a chemical to mount the response.

Four distinct types of allergic mechanisms have been described (Gell and Coombs, 1975), all of which lead to inflammatory processes. Type I hypersensitivity is IgE mediated where specific IgE link on mast cells, a subsequent challenge to either the same or an antigenically similar chemical causes degranulation releasing bioactive substances. This type of reaction is exemplified by asthma, hay fever, food allergy or anaphylactic reactions. Type II allergy is also antibody mediated; in this case the antigen may be cell bound and reacts to specific IgG- or IgM-type antibodies. The cell may be destroyed by either complement activation or phagocytosis. Drug-induced hemolytic anemia is a good example of such a reaction. Type III hypersensitivity requires a complex formation of the antigen with IgG or IgM antibodies, the immune complex may trigger complement activation leading to inflammatory changes, such as in Arthus reaction (localized edema or infiltration resulting in abscess or gangrene). The type IV hypersensitivity is cell mediated, where sensitized T helper lymphocytes react with the antigen, resulting in activation of macrophages and release of inflammatory mediators.

## Cooperation and interactions between immune and other systems

### *Immune mechanisms subsequent to pathologic damage*

The purpose of the immune functions is to ward off undesirable organisms or materials from the body, its relationship with inflammatory processes and pathologic damage is implied. Many of the cellular components of the immune system are also primary components in inflammation. Therefore it is natural that various components of this system are activated after damage to other tissues or organs of the body. The immune system responds to altered proteins or to other biological molecules formed by binding with reactive intermediates, which may be produced in the body subsequent to metabolic activation of a number of exogenous chemicals or due to tissue damage.

### *Control of the immune system by nervous system*

The immune system interacts with other systems and is profoundly influenced by the central nervous system, both directly via innervations of lymphatic organs and indirectly via neuroendocrine mechanisms. The autonomic nervous system directly innervates thymus, spleen, lymph nodes, bone marrow and other lymphoid tissues. Pharmacological manipulation of post-ganglionic noradrenergic nerve fibers affects immune functions. Neurotransmitters such as acetylcholine affect the function of immune cells and many immune cells have the ability to synthesize acetylcholine. Moreover, most immune cell types, including T cells and macrophages, express nicotinic acetylcholine receptors; activation of

nicotinic acetylcholine receptors by nicotine/nicotine-containing substances (e.g., cigarette smoke) or acetylcholine suppress inflammatory and adaptive immune responses (Sopori, 2002). The cells of the immune system produce factors that influence the nervous system.

### *Endocrine mechanisms*

Hormones, such as somatotrophin (growth hormone) and thymosin (thymic maturation factor), stimulate the immune responses, whereas steroids, including sex hormones, generally suppress the immune responses. Role of stress on immune functions has been shown; it is presumed that exposure to exogenous chemicals may induce stress. Stress-like effects after stimulation of the hypothalamic-pituitary-adrenal axis lead to cortisol production; the latter is a well-known immunomodulatory agent.

### *The complement system*

A complex system that consists of a cascade of serum proteins, mostly of  $\beta$ -class globulins, is present in most vertebrates. The function of this system is to enhance the action of antibodies by causing cell membrane alterations, often cytolysis. The complement combines with the Fc receptors of an antibody molecule and is enzymatically activated to a cytotoxic membrane attack complex. Immunotoxic chemicals may modify the complement function and may alter the inflammatory processes.

## IMMUNOTOXIC AGENTS

The structural and functional integrity of the immune system are crucial in performing its protective role against pathogenic agents. Thus, any chemically induced perturbation of the host's immune system can compromise its protective capacity and may lead to adverse health consequences for its host. A chemical substance should be considered immunotoxic when the undesired events of the chemical are: (1) a direct and/or indirect action of the xenobiotic (and/or its biotransformation product) on the immune system and (2) an immunologically based host response to the compound and/or its metabolite(s), or host antigens are modified by the compound or its metabolite(s). There is limited information on the immunotoxic effect of various chemicals in animals of economic importance. Most of the available data relate to either laboratory animals or are obtained from epidemiological studies in people.

### *Mycotoxins*

Mycotoxins are secondary metabolites of fungi and, if consumed through food or feed, are potentially



hazardous to human and animal health. Mycotoxins present a large spectrum of toxic effects; nevertheless, impairment of the immune response is a common feature of these food contaminants (Oswald *et al.*, 2003; Meisssonier *et al.*, 2008). In recent years there has been increasing awareness of the hazards imposed on both human and animal health by mycotoxins present in food and feed. Five classes of mycotoxins are of major concern in animal husbandry, namely aflatoxins, fumonisins, ochratoxins, trichothecenes and zearalenone. Due to their diverse structure these fungal toxins are able to cause a variety of symptoms in animals. The chemical diversity of mycotoxins and the wide range of matrices in which they can be found pose great challenges to analytical chemists. Therefore, a need exists for a reliable, economical and easy-to-use assay for the measurement of the mycotoxin content, especially in the raw materials for food and feed production (Cozzini *et al.*, 2008). Mycotoxins such as aflatoxin B<sub>1</sub> and fusarium T-2 toxin inhibit protein synthesis and cell proliferation, and some may have selective effects on various subpopulations of lymphocytes (Girish *et al.*, 2010). Several mycotoxins are cytotoxic to lymphocytes *in vitro*, perhaps because of their effects on membranes or interference with macromolecular synthesis and function. Selected effects of immunotoxic mycotoxins that have been studied in domestic animals are listed in Table 23.4.

Aflatoxin B<sub>1</sub>, a mycotoxin produced by *Aspergillus flavus* or *A. parasiticus*, is a frequent contaminant of food and feed. Aflatoxin B<sub>1</sub> exhibits immunosuppressive properties; ingestion of contaminated feeds increases susceptibility to infection (Joens *et al.*, 1981; Venturini *et al.*, 1996). Aflatoxin B<sub>1</sub> mainly exerts its effects on cell-mediated immunity (Bondy and Pestka, 2000; Wada *et al.*, 2008). It decreases lymphoid cell populations, especially the circulating activated lymphocytes, suppresses lymphoblastogenesis and impairs both cutaneous delayed-type hypersensitivity (DTH) and graft versus host response. Aflatoxin B<sub>1</sub> also decreases NK-mediated cytotoxicity and several macrophage functions, such as phagocytic activity, intra-cellular killing or production of oxidative radicals (Ghosh *et al.*, 1991). Effects of aflatoxin B<sub>1</sub> are primarily on the cell-mediated immune functions; however, T cell-dependent humoral responses are also adversely affected. Although many data on the effect of aflatoxins on immune cell functions are available, few studies have investigated their effects on cytokine expression and the results are somewhat conflicting. While *in vitro* treatment of human monocytes with aflatoxin B<sub>1</sub> on LPS activation led to decreased expression and release of IL-1 $\alpha$ , IL-6 and TNF- $\alpha$  (Rossano *et al.*, 1999), swine alveolar macrophages did not respond similarly to LPS (Liu *et al.*, 2002). *Ex vivo* analysis of peritoneal macrophages from mice orally exposed to aflatoxin B<sub>1</sub> indicated an increased expression of IL-1 $\alpha$ , IL-6 and

TNF- $\alpha$  (Dugyala and Sharma, 1996). Macrophages engulf large amounts of aflatoxin-bound macromolecules, which might explain their selective sensitivities to the toxin. Blood lymphocytes from pigs fed aflatoxin B<sub>1</sub> contaminated diet, when stimulated *in vitro* with mitogens, exhibited decreased expression of proinflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) and increased expression of the anti-inflammatory IL-10 (Marin *et al.*, 2002; Meisssonier *et al.*, 2008). These results suggest that aflatoxin B<sub>1</sub> may depress many aspects of humoral and cellular immunities.

Fumonisins are well-known mycotoxins produced by *Fusarium verticillioides*, *F. proliferatum* and other *Fusarium* species. Different types of fumonisins have been structurally identified. The "B" series include the most prevalent type, the fumonisin B<sub>1</sub> (FB<sub>1</sub>), and it is estimated that toxin contaminates 59% of corn and corn-based products (Haschek *et al.*, 2001). Recently, fumonisins B<sub>2</sub> and B<sub>4</sub> were shown to be produced by *Aspergillus niger* isolated from coffee and grapes. Fumonisin B<sub>2</sub> was detected in coffee beans, wine and beer, adding to the list of foodstuffs that fumonisins contaminate (Scott, 2011). Consumption of food or feed contaminated with fumonisin B<sub>1</sub> can lead to disease in humans and animals. Fumonisins can cause liver and kidney toxicity, carcinogenesis, pulmonary edema and immunosuppression (Missmer *et al.*, 2006; Marin *et al.*, 2010). Their mode of action is not completely understood but they are known to influence the sphingolipid metabolism through the inhibition of ceramide synthase (Desai *et al.*, 2002). As fumonisins enter the body following consumption of contaminated food/feed, the intestine will be the first organ exposed to these toxins and negative effects of these toxins on the gastro-intestinal tract have been reported (Bouhet and Oswald, 2007). There have been a number of investigations involving fumonisin B<sub>1</sub> and immune responses (Bondy and Pestka, 2000; Devriendt *et al.*, 2009); and the toxins may affect mucosal immunity to pathogens. Several studies demonstrated that fumonisin B<sub>1</sub> affects innate immunity, as well as humoral and cellular responses of the acquired immunity (Bhandari and Sharma, 2002; Bouhet and Oswald, 2005; Marin *et al.*, 2007, 2010; Missmer *et al.*, 2006; Theumer *et al.*, 2002). Exposure to fumonisin B<sub>1</sub> was reported to cause localized activation of the cytokine network, suggesting that the toxin-induced changes in innate immune responses may be important in its immunotoxicity (Bhandari and Sharma, 2002; Bouhet *et al.*, 2006).

Ochratoxin A (OTA) is an immunosuppressive mycotoxin, produced by toxigenic species of *Aspergillus* and *Penicillium* fungi. Contamination of food by this mycotoxin takes place primarily during pre-harvest periods, and may contaminate any type of food. In addition, its chemical stability at high temperatures and during industrial food processing makes OTA one of the most

TABLE 23.4 Selected examples of immunotoxic effects of mycotoxins in domestic or food animals

Mycotoxins	Species	Observed effects	Reference
Aflatoxin B <sub>1</sub>	Swine	Decreased lymphocyte proliferation, macrophage migration, DTH and titers to SRBC	Oswald <i>et al.</i> (2005); Taranu <i>et al.</i> (2010)
	Bovine	Decreased lymphocyte proliferation <i>in vitro</i>	Paul <i>et al.</i> (1977)
	Chicken	Inhibitory effects on the function of PMNs Decreased cell mediated immunity and titers	Wada <i>et al.</i> (2010) Verma <i>et al.</i> (2004)
Deoxynivalenol	Swine	Activation of immune response Increased susceptibility to <i>Salmonella</i>	Malovrh and Jacovac-Strajn (2010) Vandenbroucke <i>et al.</i> (2009)
Fumonisin B <sub>1</sub>	Chicken	Decreased spleen cell viability and mitogenic response	Keck and Bodine (2006)
Ochratoxin	Swine	Reduced lymphocyte levels and apoptotic phagocytes	Müller <i>et al.</i> (1999)
	Chicken	Decreased cell-mediated immunity and titers	Verma <i>et al.</i> (2004)
Patulin	Rabbit	Reduced lymphocyte proliferation and serum immunoglobulins	Escoula <i>et al.</i> (1988a, b)
T-2 toxin	Swine	Impair acquired immune response	Meissonnier <i>et al.</i> (2008)
	Bovine	Reduced peripheral blood mononuclear cells proliferation	Charoenpornsook <i>et al.</i> (1998)
Zearalenone	Chicken	Modulate immune response to coccidial infections	Girgis <i>et al.</i> (2010)
	Swine	Divergent effects on innate immunity and cell proliferation	Marin <i>et al.</i> (2010)

abundant food contaminating mycotoxins. Due in part to its long serum half-life in man, almost 100% of all human blood samples from some geographic regions are positive for OTA. The immunosuppressive activity of OTA is characterized by decreased size of secondary lymphoid organs, such as the thymus, the spleen and the lymph nodes. OTA suppresses the antibody responses, alters the number and functions of the immune cells and modulates cytokine production (Müller *et al.*, 1999). The immunotoxic activity of OTA probably results from apoptotic and necrotic cell death of the immune cells together with their slow replacement due to the inhibition of protein synthesis (Al-Anati and Petzinger, 2006).

Patulin is produced by several *Penicillium*, *Aspergillus* and *Byssachlamys* species. Inconsistent reports on patulin toxicity in various species have created an unclear picture of potential harmful effects of patulin on the immune system (Escoula *et al.*, 1988a, b). Earlier studies in mice showed that patulin increased the number of splenic T cells, lowered serum Ig levels, depressed the DTH response, increased neutrophil count and increased the resistance to *Candida albicans*. On the other hand, patulin administration in mice and rabbits resulted in reduced lymphocyte blastogenesis and suppressed chemiluminescence response of peripheral leukocytes (Escoula *et al.*, 1988a; Sharma, 1993).

Trichothecenes, particularly deoxynivalenol (DON), are the most commonly encountered *Fusarium* mycotoxins (Pestka and Smolinski, 2005). The mechanism, human exposure and toxicological relevance of DON have been recently reviewed by Pestka (2010). The immunomodulatory effects of trichothecenes have been found to vary between immunosuppression and immunostimulation, depending on the dose, duration and time of exposure (Bondy and Pestka, 2000).

Trichothecene stimulates the immune system at low doses, but suppresses at high doses, mainly due to leukocytosis (Pestka, 2003; Desjardins, 2006). Repetitive exposure to high doses of trichothecenes may result in the injury to actively dividing cells in the bone marrow, intestinal mucosa, spleen and thymus (Desjardins, 2006). The ability to inhibit protein synthesis has been described as a major effect of trichothecenes (Bondy and Pestka, 2000; Desjardins, 2006). Low-dose trichothecene exposure transcriptionally and post-transcriptionally up-regulates expression of cytokines, chemokines and inflammatory genes with concurrent immune stimulation, whereas high-dose exposure promotes leukocyte apoptosis with concomitant immune suppression. DON and other trichothecenes, via a mechanism known as the ribotoxic stress response, bind to ribosomes and rapidly activate mitogen-activated protein kinases (MAPKs). The latter are important transducers of downstream signaling events related to immune response and apoptosis (Pestka *et al.*, 2004). T-2 toxin causes acute and chronic toxicity, and induces apoptosis in immune cells and fetal tissues (Li *et al.*, 2011). T-2 and DON cause severe depletion of T cells and are immunosuppressive *in vivo* and *in vitro*. Although immunosuppressive in mice, paradoxically T-2 toxin increased the expression and production of IL-12, TNF- $\alpha$ , IFN- $\gamma$  and IL-2, but decreased IL-1 $\beta$  in a concentration-dependent manner in peritoneal macrophages and lymph nodes (Ahmadi and Riazipour, 2008). On the other hand, DON increased the production of both TNF- $\alpha$  and IL-1 $\beta$  by macrophages (Yang and Pestka, 2002; Döll *et al.*, 2009). Thus, the major immunopathological effects of trichothecenes might be related to their proinflammatory responses. Other mycotoxins found as food and feed contaminants may cause similar changes in the immune system.

TABLE 23.5 Selected metals evaluated in domestic or food animals for immunotoxic potential

Metals	Reported immunotoxic effects	Reference
Lead	Immunosuppression in cattle Decreased peripheral lymphocyte counts and depressed interferon $\gamma$ -like cytokine by splenocytes of chicken	Black <i>et al.</i> (1992) Lee <i>et al.</i> (2001)
Mercury	Suppressed immunocompetence in fish	Sweet and Zelikoff (2001)
Molybdenum	Decreased antibodies to swine erythrocytes in calves	Gengelbach and Spears (1998)
Copper	Higher titers to ovalbumin in heifers	Salzer <i>et al.</i> (2004)
Zinc	Reduced phagocytic activity of hemocytes Decreased antibody response but increased phagocytosis by peritoneal exudates cells in pigs Higher antibody titers to ovalbumin in heifers	Mottin <i>et al.</i> (2010) Van Heugten <i>et al.</i> (2003) Salzer <i>et al.</i> (2004)
Chromium	Increased antibody response to ovalbumin in calves Decreased lymphocyte proliferation and phagocytosis in carps	Chang <i>et al.</i> (1996) Steinhagen <i>et al.</i> (2004)
Selenium	Enhanced cell-mediated immune response in eiders	Wayland <i>et al.</i> (2002)
Vanadium	Increased IL-6 activity in macrophage supernatants	Qureshi <i>et al.</i> (1999)
Cadmium	Immunostimulation, induction of autoantibodies	Ohsawa (2009)

## Metals

The industrial activities from the last century have resulted in massive increases in our exposure to toxic metals such as lead, cadmium, mercury and arsenic; these are now present in the entire food chain. A few selected studies with metals involving domestic animals are listed in Table 23.5. Lead has been shown to impair immune function and/or host resistance to disease dating back to the 1960s. However, it is only in recent years that lead has been recognized as a new category of immunotoxicants that dramatically shift immune function without significantly affecting the number or sub-type distribution of immune cells in lymphoid organs (Dietert and Piepenbrink, 2006). Lead is of particular concern in veterinary practice as exposure and poisoning of domestic animals with this metal has been reported (Black *et al.*, 1992). Lead, mercury and nickel are immunosuppressive; however, low doses of selenium, zinc and vanadium are immunostimulatory (Qureshi *et al.*, 1999; Wayland *et al.*, 2002; van Heugten *et al.*, 2003). Mercury is ubiquitously present in the environment resulting in permanent low-level exposure in human populations. Mercury can be encountered in three main chemical forms: elemental, inorganic and organic, which can affect the immune system in different ways. Subtoxic doses of mercury induce highly specific autoantibodies and a generalized activation of the immune system (Vas and Monestier, 2008; Sheir *et al.*, 2010). Some reports show that lead and cadmium stimulate the production of cytokines and IgE antibodies that may explain the increased number of atopic diseases in populations exposed to these two metals (Skoczynska *et al.*, 2002).

Immunotoxic effects of heavy metals have been recently reviewed and mechanisms may be diverse

(Ohsawa, 2009). Metals may chelate biological macromolecules and may affect protein synthesis, membrane integrity and/or nucleic acid replication. While *in vitro* studies may unravel potential molecular mechanisms, they have little value in predicting the *in vivo* immunotoxicity and are often opposite to the immunological effects seen *in vivo*. In general, heavy metals may affect the number of circulating B cells, T lymphocytes, NK cells, memory cells and inflammatory responses.

## Pesticides

Organophosphorus (OP) pesticides are used extensively to control agricultural and household pests. These pesticides constitute a diverse group of chemical structures exhibiting a wide range of physicochemical properties. The primary toxicological action of OP arises from its inhibition of the enzyme acetylcholinesterase that degrades the neurotransmitter acetylcholine. The main toxicity of OP pesticides is neurotoxicity (Pope, 1999; Bajgar, 2004), but there is little consensus that OP pesticides are immunotoxic (Sharma, 2006). It has been reported that OPs affect the function of neutrophils (Hermanowicz and Kossman, 1984) and macrophages (Crittenden *et al.*, 1998), and suppresses antibody production (Johnson *et al.*, 2002) in animals and humans. There are very few papers that deal with the mechanism of OP-induced immunotoxicity (Galloway and Handy, 2003; Langley *et al.*, 2004; Li, 2007). Rabbits fed low doses of methylparathion were reported to have atrophy of the thymus cortex and fewer antibody-forming cells in lymph nodes; splenic morphology was also considerably altered. Diets containing cabaryl or carbofuran fed to rabbits reduced the numbers of activated lymphocytes in

lymph nodes, decreased the number of splenic germinal centers and produced atrophy of the thymic cortex. The immunosuppressive effect of carbaryl containing feed is also reported in chicken which includes reduced macrophage function and lymphocyte proliferation (Singh *et al.*, 2007).

Although malathione exposure did not affect the innate immune response, it suppressed humoral immune responses in birds (Nain *et al.*, 2011). Humoral immune response against *Salmonella typhimurium* infection was also decreased in rabbits exposed to malathion or dichlorvos. A single dose of malathion, parathion or dichlorvos at the concentration that causes cholinergic distress reduced the primary IgM response to sheep red blood cells (Johnson *et al.*, 2002).

The functional status of the immune system in female mice exposed to a single oral dose of dimethoate (16mg/kg) was evaluated by assessing cell-mediated and humoral immune responses (Aly and el-Gendy, 2000). Dimethoate decreased the spleen weight without affecting the body weight, decreased the number of erythrocyte rosette forming cells and the proliferative response of the spleen to T and B cell mitogens, i.e., phytohemagglutinin and lipopolysaccharide, respectively. Organophosphates triphenylphosphate and triphenylphosphine oxide primarily affected various parameters of innate immunity.

Various chlorinated hydrocarbon insecticides, such as dieldrin, dichlorodiphenyl-trichloroethane (DDT) and lindane, have been tested for immunotoxic effects. In animal models, DDT was shown to be immunosuppressive and decreased the host immunity against a wide range of invading pathogens (Banerjee *et al.*, 1997;

Kim *et al.*, 2004). Occupational exposures to DDT were also associated with immunotoxicity.

## Industrial and environmental chemicals

Animals are constantly exposed to a wide variety of foreign chemicals, many of which are potentially toxic and some cause clinical poisonings. Immunotoxic effects of common environmental chemicals are summarized in Table 23.6. Increasing use of chemicals such as brominated and chlorinated flame retardants have caused environmental contamination globally and may have adverse health effects in animals and humans. In 1973, polybrominated biphenyl (PBB) accidentally entered the food chain in Michigan, when Firemaster FF-1, a commercial flame retardant, was inadvertently added to cattle feed. Following the Michigan accident, a large number of animal studies showed that PBB causes immunotoxic effects in cattle and laboratory animals, including atrophy of various lymphoid organs and depressed antibody responses. On the other hand, cattle inadvertently exposed to PBBs (accumulating up to 30ppm PBB in body fat) showed no adverse immunological effects (Kateley *et al.*, 1982); however, spleens of the mink exposed to 10ppm of DE-71 exhibited increased germinal center and B cell hyperplasia (Martin *et al.*, 2007). Chlorinated phenols, found in a variety of foods, were also evaluated for immunotoxicity (Forsell *et al.*, 1981). PCBs are members of the halogenated aromatic hydrocarbon class of chemicals that include the prototype 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic synthetic chemical known to date. TCDD and

TABLE 23.6 Selected examples of contaminants in feed that may influence immune responses in animals

Chemicals	Species and effects	Reference
Organochlorine insecticides	Accelerates the development of systemic lupus erythematosus in ovariectomized mice	Wang <i>et al.</i> (2007)
Organochlorine insecticide mixture	Elevated lymphocyte proliferation and leukocyte phagocytic activity after exposure in swine	Bilrha <i>et al.</i> (2004)
Polyaromatic hydrocarbon mixture	Enhances components of innate immunity in resident mummichogs, while actively suppressing humoral immune responses	Frederick <i>et al.</i> (2007)
Polychlorinated biphenyls	Decreased antibody titers and lower concentrations of IgG, IgM in blood, negatively affect the immune system of the glaucous gull chick	Sagerup <i>et al.</i> (2009)
Polychlorinated biphenyls	Decreased antibody titers, decreased thymus and bursa cellularity, suppressed antibody responses in juvenile chickens following an <i>in ovo</i> exposure	Lavoie and Grasman (2007)
Polychlorinated biphenyls and other contaminants	Decreased NK cell activity, T cell mitogen response, antigen-specific proliferation, mixed lymphocyte response and delayed-type hypersensitivity in harbor seals	Van Loveren <i>et al.</i> (2000)
Tributyltin and polychlorinated biphenyls	Suppressed antibody response and phagocyte oxidative burst activity in catfish	Regala <i>et al.</i> (2001)



other dioxin-like compounds suppress humoral immunity, mainly by interfering with B cell differentiation and antibody production (Dooley and Holsapple, 1988; Tucker *et al.*, 1986; Sulentic and Kaminski, 2011). Dioxin-like compounds also disrupt the development and maintenance of the acquired immunity (Zhang *et al.*, 2010). Atlantic bottled-nose dolphin and Baltic harbor seal populations have been found to be highly susceptible to immunosuppression by PCBs.

A number of other chemicals, particularly those used in the plastics industry, such as bisphenol A, may impair the immune response (Richter *et al.*, 2007). Inhalation of toluene diisocyanate vapor induces allergic rhinitis in mice (Johnson *et al.*, 2007). Immunotoxic effects of 20 different industrial chemicals, such as vinyl chloride, benzene and styrene, are reviewed by Veraldi *et al.* (2006).

Other chemicals and drugs

Many drugs have the capability to alter the function of the immune system and, therefore, are used therapeutically. On the other hand, several drugs may have immunotoxic side effects. Diethylstilbestrol (DES), dexamethasone (DEX) and cyclophosphamide are immunosuppressive chemicals and induce similar thymic pathophysiology; however, the mechanism of immunotoxicity of these compounds is not overlapping (Patterson and Germolec, 2006). DES is a synthetic estrogen and commonly used as a growth promoter in animals, suppresses both cell mediated and humoral immunity, and is a potent macrophage activator. Moreover, prenatal exposure to DES may alter the development and function of the immune system. Recent reports provide little support for the association between prenatal DES exposure and development of autoimmune diseases

(Strohsnitter *et al.*, 2010). Cyclophosphamide, a potent immunosuppressive agent and protein synthesis inhibitor, impairs both humoral and cell-mediated immune responses. In mammals, cyclophosphamide suppresses graft versus host reaction, depletes leukocyte subpopulations, and inhibits T and B cell mitogenesis.

TESTING FOR IMMUNOTOXICITY

The realization that environmental chemicals might adversely affect the immune system has raised serious public concern. There is no single or simple test that can be used to evaluate the immunotoxic effects of a chemical. We have listed some commonly employed immunotoxicity tests in Table 23.7. Because of the complexity of the immune system, the initial strategy to evaluate immunotoxicity of an agent is to apply a tiered panel of assays. The first tier is a general screen for immunotoxicity and, depending on the testing agency, may or may not include functional tests such as the measurement of antibody responses after *in vivo* antigenic challenge, NK cell activity or non-specific immune parameters (e.g., lymphoid organ histology, serum immunoglobulin levels). The second tier usually consists of tests that identify specific target cells and examines resistance to infectious agents or neoplastic potential. Several studies have shown that assessment of only a minimal number of appropriate tests may be needed to predict immunotoxicity successfully in rodents. In particular, assessment of humoral immunity, via quantitation of antigen-specific T-dependent antibody responses, has been shown to be the best single indicator of potential immunotoxicity of

TABLE 23.7 Clinical or toxicological tests indicative of compromised immunologic responses

Tests	Basic principle
Hematology and lymphatic organ weight and pathology	Immunotoxic potential may be derived on general toxicity studies by total and differential leukocyte counts, and when spleen, thymus and lymph nodes are examined.
Lymphocyte proliferation	Responses to T and B cell mitogens, either on cells derived from exposed animals or cells treated <i>in vitro</i> .
Lymphocyte phenotyping	Phenotyping of lymphocytes bearing different cell surface markers can be used in routine studies.
Antibody production	Antibody titers to commonly used vaccinations are useful. In laboratory animals antibodies against T cell-dependent or -independent responses can be employed. Both antibody titers and plaque-forming cells assay have been used.
Delayed-type hypersensitivity responses	These T cell-dependent responses are commonly employed in various smaller species; however, they can also be used in large animals.
Phagocytosis and cell migration	Leukocytes and macrophages can be evaluated for phagocytic activity and chemotaxis; production of various signaling molecules can be measured.
Cytokine/chemokine levels	Measuring levels of various cytokines/chemokines in serum, BAL fluid and/or in tissues is useful after toxicant exposure.
Resistance to pathogens	Bacterial, viral or parasitic infections that have known effects on immune parameters can be used. Mortality to sublethal infections can be of use in early stages.

a compound (Luster *et al.*, 1988). A potentially highly useful parameter to predict immunotoxicity may be cytokine production. Cytokines regulate a variety of immunological responses, including inflammation, apoptosis, hematopoiesis and adaptive immunity (Corsini and House, 2010). There is a growing belief that *in vitro* models of immune response would eventually eliminate the use of animals in biosafety testing (Corsini and Roggen, 2009). Relevant *in vitro* models for hypersensitivity, immunosuppression, immune activation and autoimmunity will be needed to bypass animal use for immunotoxicity testing. A great challenge will be to simulate multiple organ interaction in eliciting an immune response to a chemical in non-animal models. Current *in vitro* immunotoxic tests, in most instances, do not replicate *in vivo* results.

## CONCLUSIONS AND FUTURE DIRECTIONS

A major issue facing toxicological science today is how to evaluate the toxicological data from *in vitro* and animal models and define the safety levels for human exposure. The immunosuppressive potential of a chemical is of little use if the effects on the immune system occur at doses that are significantly higher than the toxic doses for other systems. Understanding the mechanism(s) of action and the structure–activity relationships might help in defining the immunotoxic potential of a chemical. Effects on profiles offer a strong promise to define the potential immunotoxicity of a chemical. Recent expansion in the knowledge of cytokine biology and the realization that cytokines play an important role in diseases have created a need for precise measurements of cytokines in various body fluids, tissues and cells. Cytokine analysis and quality control issues need to be resolved for the routine use of this parameter in evaluating immunotoxicity (Corsini and House, 2010).

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# Toxicity of nanomaterials

*Deon van der Merwe and John A. Pickrell*

## INTRODUCTION

The toxicology of nanomaterials has emerged as a challenging field of investigation because the widely applied toxicological paradigm “the dose makes the poison” appears to break down when the toxicological effects of materials are investigated at the nanometer scale. At this scale, the physical–chemical characteristics of materials are highly dependent on unit size and shape, and their interactions with biological systems may differ markedly from chemicals in solution, and from larger particles. The mass of nanomaterial to which susceptible tissues are exposed therefore becomes relatively less important as a determinant of the degree of biological impact compared to the size and form of the material. Particles, fibers, rods or tubes with one or more dimensions <100 nm are generally referred to as nanomaterials, or more specifically as nanoparticles (also known as ultrafine particles), nanofibers, nanorods or nanotubes. Nanomaterials are formed in nature during combustion, geological processes and biological processes. They are also commonly produced unintentionally by man in processes such as power generation from coal, the combustion of fuels in automobiles and many other industrial, engineering and domestic activities. Additionally, and at a rate that is rapidly accelerating, nanomaterials are manufactured intentionally, through molecular-level engineering, to obtain materials with unique mechanical, optical, electrical, magnetic and chemical properties (Tsuji *et al.*, 2006; Richards, 2009). These materials can be referred to as engineered nanomaterials (ENMs). They form the core of a rapidly growing industry where ENMs are incorporated into a wide variety of manufactured products. The

increasing use of ENMs, and the uncertainties associated with their unusual and often poorly understood biological effects, creates concern regarding their potential for causing unanticipated adverse health effects.

Investigators continue to discover unique properties of materials at the nanometer scale (Hoet *et al.*, 2004). Commercialization of products that exploit these unique properties is increasing, and have already found commercial application in sporting goods, tires, sunscreens, sanitary ware coatings, stain resistant textiles and clothing, food products and electronics (Hoet *et al.*, 2004; Nel *et al.*, 2006; Tsuji *et al.*, 2006). The list of products incorporating nanotechnology is expected to continue to expand indefinitely. By some estimates, growth of nanotechnologies and use of ENMs will far exceed the impact of the industrial revolution; they are predicted to become a \$1 trillion market by 2015 (Nel *et al.*, 2006). However, in spite of the seemingly unstoppable success of this technology, the unique properties of ENMs also present considerable new challenges to understanding, predicting and managing potentially adverse health effects following exposure. Technological development and applications are outpacing research for safe use and documentation of health and environmental risk (Hoet *et al.*, 2004; Tsuji *et al.*, 2006). Although exposure to ENMs is often intentional and controlled, widespread use of increasing quantities of ENMs increases the likelihood of uncontrolled human and animal exposure to nanomaterials in quantities that may result in biological effects. The future success of nanotechnology will be influenced by the level of public acceptance of the risks from exposure to ENMs, relative to the benefits. The increased demands for development to obtain the promised benefits from nanomaterials are balanced by an increasing

need for a proactive approach in estimating the environmental and health costs (Ngo *et al.*, 2008).

Concerns regarding the suitability of traditional toxicological assessment methods for evaluating ENMs gave birth to a relatively new discipline, named nanotoxicology. The discipline can be defined as: “the study of the adverse effects of ENMs on living organisms and ecosystems, including the prevention and amelioration of such adverse effects” (Oberdörster, 2010). As with conventional chemicals, risk assessment will be the basis of assessing and regulating exposure to nanomaterials to protect human, animal and environmental health. Many applications will likely have limited, or at least manageable, exposures and effects, and are therefore expected to cause insignificant adverse health effects; however, these assumptions may be difficult to substantiate based on traditional assessment methods because the effects of ENMs may be unique, product specific and unpredictable from data derived from larger particles or from chemicals in solution (Hoet *et al.*, 2004; Tsuji *et al.*, 2006). Studies have shown that some ENMs are not inherently benign, and that they may distribute throughout the body, inducing inflammation, oxidative stress and other adverse effects (Nel *et al.*, 2006). The results of studies demonstrating ENM adverse effects should, however, be interpreted with caution, especially when very high experimental doses are used that may not represent realistic exposure scenarios. Meaningful risk assessments should address questions related to the identification of hazards, exposure assessment and toxicokinetics including persistence in cells and subcellular structures (Oberdörster, 2010). Assuming public acceptance, toxicologists’ experience with past “miracle” materials advises us that caution in using novel substances without fully evaluating potential health risks may be ill advised. The discovery of serious adverse health effects, such as asbestos-like effects associated with multiwalled carbon nanotubes, has prompted nanotoxicologists to recommend heightened caution in the release of ENMs until more adequate information becomes available. Moving forward, the field of nanotoxicology is faced with many challenges, including the need to modify our understanding of the concept of “dose,” which, at the nanoscale level, is dependent on multiple physical–chemical factors other than the familiar mass or concentration used in traditional toxicology. Another significant challenge is the dynamic nature of the physicochemical characteristics of materials at the nanoscale, which often change over time, and as a consequence of interaction with biological systems (Maynard *et al.*, 2011). The specific changes that occur and their implications for biological interaction depend on the specific nanomaterial. Metal oxide nanoparticles, for example, may aggregate and agglomerate without significant reduction of the total surface area of the agglomerate, but it may limit interaction with internal surfaces of the agglomerate

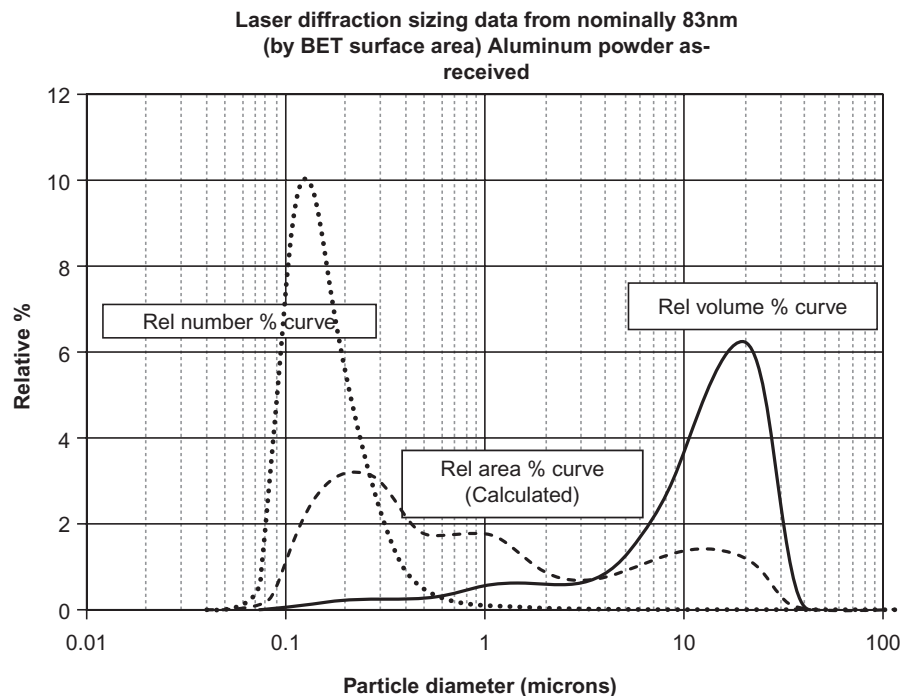
to substances that are small enough to penetrate the agglomerate (Pickrell *et al.*, 2010). Thus, unique effects, associated with repeated and combined exposures, are expected to emerge.

## NANOMATERIALS CHARACTERIZATION

The unusual physicochemical properties of ENMs are attributable to their morphology, composition and surface characteristics. In an effort to standardize the description of nanomaterials in a way that will reduce uncertainty in data interpretation and facilitate cross-comparison between studies, the Minimum Information on Nanoparticle Characterization (MINChar) initiative (MINChar Initiative, 2008) identified a list of parameters that should ideally be characterized during studies. The parameters include particle size and size distribution, agglomeration state and aggregation, particle shape, chemical composition, crystal structure, surface composition, purity and levels of impurities, surface area, surface chemistry (including reactivity and hydrophobicity) and surface charge. It was also recognized that the characteristics of ENMs may not be stable during storage, handling, preparation and delivery into biological systems. Since the media used for delivery may change ENM properties, particular emphasis should be placed on characterization of ENMs “as administered.” It was also recommended that responses should be interpreted against a range of dose metrics, including mass, surface area and number concentration.

Particles or particle agglomerates are usually not perfect spheres and are almost never singly dispersed. However, particle size is most often defined as the diameter of a sphere that is equivalent in the selected property to the particle measured; this makes it possible to conveniently plot size distributions of irregularly shaped particles or particle-agglomerates using a single value (diameter) along a single axis. The property most often described is volume or mass; that is, the diameter of a sphere of equal volume to the particle(s) in question (volume or mass diameter). In Figure 24.1, particle diameter is expressed as that which was the median (120 nm), surface area (250 nm) or volume (20  $\mu\text{m}$  (20,000 nm)). Peak distributions in this figure contain some agglomerates of smaller particles.

The changes in ENM surface characteristics following delivery into a biological system are of particular importance when biological interactions are assessed. Proteins attach selectively to nanoparticle surfaces to form nanoparticle-protein coronas. This process, which depends on the adsorption properties of nanomaterial surfaces (Xia *et al.*, 2010), can dramatically change particle surface



**FIGURE 24.1** Laser diffraction size data for “nanoscale” aluminum powder used for *in vitro* toxicity experiments. Note the apparent difference in size when depicted as a number distribution versus an area or volume distribution. This occurs because volume scales as the cube of the particle diameter and calculated area scales as the square. Each curve, if presented alone, would give an incomplete picture of the particle size distribution/state of agglomeration of the sample. The three curves will overlay only for an ideal spherical, monodisperse, unagglomerated system. By comparing changes in particle size distribution to that of an “ideal” dispersion, a qualitative assessment of the degree of agglomeration can be made. (Reproduced with permission of Powers *et al.*, 2006.)

characteristics and, consequently, interactions with biological systems. Combination with biological macromolecules may both detoxify nanomaterials, and promote uptake into cells that can reduce clearance and lead to chronic, degenerative changes.

Solubility can be an important determinant of biological response. Solubility is a function of chemical activity, specific surface area, radius and curvature, agglomeration and specific chemicals that are adsorbed to the ENM (Borm *et al.*, 2006). Larger, more densely aggregated particles will dissolve more slowly. Adsorbed organic chemicals will slow solvation (Powers *et al.*, 2006). Solubility of ENMs is inversely correlated with their biopersistence. Since solubility and chemical reactivity are strongly dependent on the physical-chemical environment; they should be measured in an environment as near to that of the target biological environment as possible. This can sometimes be accomplished with *in vitro* conditions closely simulating biological solutions. Specific interactions may be present in biological fluids that are absent in simple solvents. For example, when determining solubility and reactivity in the lung, it is important to consider not just solubility in water, but in fluids that bear significant resemblance to that of the epithelial lining fluid (ELF). A minimal ELF volume is predicted to be about 40 to 100 ml for mature humans. The pH varies from 6.9 at the end of inspiration, to 7.5 at the end

of expiration. Bicarbonate acts as a buffer, and the change in pH is caused by removing CO<sub>2</sub> of expiration (Langmuir, 1965). Bicarbonate concentration in lung simulant fluid increases the solubility of MgO (Pickrell *et al.*, 2009). Surprisingly, macrocrystalline (bulk) MgO is dissolved as extensively as nanosized MgO in lung simulant fluids in about 10–20 minutes. This paradoxical result suggests that bicarbonate chemical activity may have a direct relation to the rapid dissolution of MgO in lung simulant fluid related to its modest solubility in distilled water. Stoichiometry of the likely chemical species suggests a conversion of MgO to Mg(OH)<sub>2</sub> in aqueous media and a subsequent conversion to the hydrated carbonate. These phenomena serve to illustrate the potential error associated with studies conducted in simple solutions when attempting to predict activity in complex biological systems.

## ANIMAL OR TISSUE EXPOSURES

### *In vitro* exposures to nanomaterials

Early indicators for ENM-derived adverse health effects were needed for an adequate assessment cytotoxicity of different types of well-characterized ENMs; cytotoxicity



was used to infer relative risk (Brunner *et al.*, 2006). For this purpose, the authors chose a mesothelioma and a fibroblast cell line. Two assays were performed. The first was mean culture activity, indicated by mitochondrial activity in converting a formazan type of dye from its leukoform to an active dye. In addition, DNA content, indicating cell number, was measured. Other than the positive asbestos control, none had >20% of the particles larger than 200 nm. Specific surface area was also higher than the asbestos positive control (90–190 m<sup>2</sup>/g versus the 8.5 m<sup>2</sup>/g of the asbestos). Mass median sizes were 20 to 50 nm and surface area median sizes were 6 to 21 nm, although they differed in terms of the shape and degree of agglomeration (Brunner *et al.*, 2006). Soluble amorphous silicate, compared with a negative control, caused minimal response in either cell line, consistent with its anticipated lack of toxicity. Crocidolite asbestos had a significant reduction in both cell activity and cell number (DNA) in both cell lines. The mesothelioma cell line was more severely affected, perhaps reflecting its metabolic activity. This toxicity was consistent with expectations from a positive control. Calcium phosphate had no significant cytotoxicity at 3 days; these data are consistent with it having no toxicity like the negative control particle – amorphous silica. Insoluble metal oxides (TiO<sub>2</sub>, ZrO<sub>2</sub> and CeO<sub>2</sub>) had modest toxicity at 3 days of exposure, which was most prominent in mesothelioma cells. By 6 days of exposure, these cells had returned to normal. Morphologically, the high dose of zirconium oxide caused the appearance of decreased cell number and function, even after 6 days of incubation. These data are consistent with only a modest health effect relative to the positive control. Zinc and ferric oxide were slightly soluble and quite toxic to both cell lines after 3 days of incubation. The authors advance no specific explanation for zinc toxicity beyond chemical toxicity, but experimental conditions suggest considerable local particle concentrations. For iron, the toxicity resulted from free radical production from the Haber-Weiss reaction. Because this reaction occurred at 40-fold less than the chemical toxicity of iron, it is said to be specific for the nanoparticle.

*In vitro* analysis of particle size-related effects on alveolar macrophages, epithelial cells and neutrophils demonstrates size- and oxidant-dependent responses. Increased interleukin (IL)-6 in cultured lung cells suggested a pro-inflammatory, oxidant response. Reduced cell numbers, especially with Mn-containing nanoparticles, suggested cytotoxicity at high doses, probably from local concentration of particles (Pickrell *et al.*, 2010). These effects were observed after *in vitro* exposure to concentrated PM (2.5) ambient particles collected in real time. Oxidative stress, mediated by ROS, is an important mechanism of particle-induced lung inflammation (Tao *et al.*, 2003).

## Intratracheal instillation and pharyngeal aspiration

### Ultrafine particles

Exposure to ambient air pollution particles caused increased cardiopulmonary morbidity and mortality, particularly with pre-existing disease (Tao *et al.*, 2003). Exacerbation of pulmonary inflammation in susceptible people (e.g., asthmatics, COPD patients) is a central mechanism by which particles cause lung disease, primarily from organic dusts (Pickrell *et al.*, 2009, 2010). Metal oxide nanoparticles may also have direct cytotoxic effects in lung cells. After intratracheal instillation, ultrafine colloidal silica (UFCS) particles had greater ability to induce tissue damage and inflammation compared to fine colloidal silica (FCS) particles. Electron microscopy demonstrated UFCS and FCS particles on both bronchiolar and alveolar wall surfaces; type I epithelial cell necrosis and basement membrane damage was greater in UFCS than FCS particles (Kaemawatawong *et al.*, 2005).

Inhalation of highly insoluble ultrafine particles (diameter 20 nm) of low intrinsic toxicity (TiO<sub>2</sub>) resulted in pulmonary inflammatory responses. However, these effects were not acute and occurred only after prolonged inhalation exposure of the aggregated ultrafine particles at high levels >1 milligram/m<sup>3</sup> (Oberdörster *et al.*, 1995). Ultrafine carbon particles are more toxic on an equal mass basis than fine carbon particles, but this is not true if they are examined on a total surface area basis (Oberdörster *et al.*, 2010).

### Carbon nanotubes

Pharyngeal aspiration of single-walled carbon nanotubes (SCWNT) elicited an acute pulmonary inflammation in C57BL/6 mice that progressed to fibrosis and granulomas (Shvedova *et al.*, 2005). SWCNT caused a dose-dependent release of protein, LDH and gamma-glutamyl transferase activities into bronchoalveolar lung washings (lavage). In addition, elevated 4-hydroxynonenal (oxidative biomarker), depleted glutathione in lungs, leukocyte accumulations and cytokines were present in lavage fluids at days 1–7. This was followed by fibrogenic transforming growth factor (TGF-β1) that peaked on day 7. The progressive fibrosis found in mice exhibited two distinct morphologies – SWCNT-induced granulomas, and diffuse interstitial fibrosis and alveolar wall thickening. Functional respiratory deficiencies and decreased bacterial clearance (*Listeria monocytogenes*) were found in mice treated with SWCNT (Shvedova *et al.*, 2005). *In vitro* exposures of multiwalled carbon nanotubes to fibroblasts at high doses induce a similar inflammation (Ding *et al.*, 2005). Equal doses of ultrafine carbon black particles or fine crystalline silica (SiO<sub>2</sub>) did not cause either

granulomas or alveolar wall thickening. Instead, they caused weaker pulmonary inflammation and damage. Inhalation of multiwalled carbon nanotubes induced no lung changes, but marginal pulmonary and systemic immunosuppression was observed (Mitchell *et al.*, 2007).

### Intranasal exposure

High doses of carbon black, given intranasally, showed adjuvant activity as indicated by enlargement of peribronchial lymph nodes and ovalbumin-specific production of thymocyte (Th) 2 specific interleukin (IL)-4, IL-5 and IL-10. Local cytokine production after carbon black exposure is predictive of allergic airway inflammation (De Haar *et al.*, 2005).

### Inhalation exposure

Both particle size and solubility are important determinants of biological response after lung exposure. If cleared rapidly they will be less injurious; if not they will remain as a chronic irritating source (Pickrell *et al.*, 2004, 2009). Particles must be small enough to be inhaled into deep lung – usually taken to be  $\leq 4\mu\text{m}$  (4000nm). Particles between 4000nm and 500nm will be phagocytized and cleared rapidly. Particles  $<500\text{nm}$  aggregate size will be less efficiently phagocytized by phagocytes and may translocate to the pulmonary interstitium and remain there for relatively long periods of time. For example, in a recent study of fullerene particles with a geometric mean diameter of 96nm (based on number) in rat lungs, it was estimated that  $>99\%$  of particles remaining in the lung 6 months after exposure were in the interstitium, while particles that did not enter the interstitium were cleared by alveolar macrophages relatively rapidly with a half-life of 15–24 days (Shinohara *et al.*, 2010).

Thermodegradation products of polytetrafluoroethylene (PTFE) contain singlet ultrafine particles (median diameter 26nm); the fumes are toxic to rats at inhaled concentrations of  $0.7\text{--}1.0 \times 10^6$  particles/ $\text{cm}^3$ . Inhalation studies with ultrafine particles ( $\leq 100\text{nm}$ ; NP) in rats suggest that particles  $\leq 50\text{nm}$  in diameter may contribute to increased mortality and morbidity. Inhalation exposure in rats caused an acute hemorrhagic pulmonary inflammation and death after 10–30min (Oberdörster *et al.*, 1995). These results confirmed reports from other laboratories that the toxicity of PTFE fumes should not be attributed only to gas-phase components of these fumes. Aging of PTFE fumes with concomitant aggregation of the ultrafine particles greatly decreases their toxicity.

Fumes from overheated Teflon<sup>TM</sup> (PTFE)-coated frying pans cause a fatal hemorrhagic pneumonitis in caged

birds directly adjacent in both time and space to the overheating (Blandford *et al.*, 1975). Birds appear to be more sensitive than rats and humans to direct inhalation of Teflon<sup>TM</sup> fumes from overheated skillets; this sensitivity probably reflects their physiologically more efficient respiratory system. When birds are removed from the overheating event by a short period of time, or when birds are in an adjacent room, there is a comparatively minimal reaction. Current understanding implies that the small particles released from heated PTFE rapidly condense into larger and less biologically potent particles (Lee and Seidel, 1991). Aged particles collected as agglomerates were not toxic when given intratracheally to rats, but the particles became toxic when rats were given inhalation exposure to fumes evolved from the reheated agglomerate. Rats died with pulmonary edema and hemorrhage reflecting Type I pneumocyte damage.

## EPIDEMIOLOGICAL DATA

Epidemiological studies show an association between particulate air pollution and acute mortality and morbidity down to ambient particle concentrations below 100 micrograms/ $\text{m}^3$ . Whether this association also indicates causality between acute health effects and particle exposure at these low levels is still unclear, but several examples of associations between particulate air pollution and adverse health effects are available, and it suggests a high probability that a cause and effect relationship exists.

### Sulfur dioxide (oxides of sulfur particles)

Sulfur dioxide (oxide of sulfur particles) comes mainly from burning sulfur-containing fuels (Sullivan *et al.*, 2006), causing both indoor and outdoor pollution. For example, poorly ventilated kerosene space heaters are sources of indoor sulfur dioxide. Sulfur dioxide particles are hygroscopic and tend to grow larger with increasing hydration of the atmosphere.

Nasal breathing filters out much of the inhaled sulfur dioxide; it is water soluble and is often absorbed in the upper portion of the respiratory tract (Sullivan *et al.*, 2006). Sulfur dioxide is irritating to the eyes, nose and airways, and its odor is detected at concentrations as low as 0.5ppm. At  $>6\text{ppm}$ , companion animals often show acute clinical signs including tearing, runny nose, cough, bronchospasm and shortness of breath. Additional chronic signs that come from prolonged exposure at lower exposure concentrations include cough, mucus hypersecretion and frequent clearing of the throat; these reflect airway inflammation and chronic bronchitis. Massive exposure is capable of inducing severe,

permanent pulmonary damage. Low-level prolonged human exposures correlate well with bronchial asthma.

### Swine barn dust exposure

Gases and/or ultrafine particles in swine barns can be important factors in the development of increased bronchial responsiveness both in animals and their human caretakers. For example, healthy human volunteers weighing pigs for three hours developed a neutrophilic pneumonitis, and they had an increased bronchial responsiveness to methacholine. Wearing a mask reduced, but did not abolish, the inflammatory response (Larsson *et al.*, 2002). *In vitro* data suggest that airway epithelial cell swine dust extract exposure enhances subsequent lymphocyte adhesion to epithelial cells by a modulation of the expression of intracellular adhesion molecule 1, and protein kinase C alpha (Mathisen *et al.*, 2004). The associated inflammation is characterized by increased numbers of neutrophils, macrophages and lymphocytes to a lesser degree, in pigs and their caretakers. The inflammation is associated with increased IL-6 in cultured cells, suggesting an asthma-like reaction (Pickrell *et al.*, 2010). Interestingly, this inflammation is most pronounced in caretakers with no prior exposure to this environment, which suggests a tolerance to endotoxin or other substances in this environment associated with repeated exposures (Von Essen and Romberger, 2003).

### Nanoparticles exposure in the home

Environmental tobacco smoke (ETS) is a health risk for companion animals. It is a major source of indoor particles, and can release more than 4000 potential toxicants into the air (Sullivan *et al.*, 2006). While the effects of smoke inhalation may be mostly due to toxic gases (e.g., carbon monoxide), ETS particles, such as acrolein, are capable of producing pulmonary damage (Fitzgerald and Vera, 2006). Frequently observed signs include wheezing, chronic cough, chronic hyperreactive airways and chronic bronchitis. Water soluble toxins are absorbed and injure mostly the upper respiratory tract, while less soluble materials go deeper into the lungs and injure the pulmonary parenchyma. The EPA has classified ETS a group A carcinogen, meaning that there is sufficient evidence to indicate that it will produce cancer in humans (Sullivan *et al.*, 2006). Epidemiological evidence in humans demonstrates a strong relationship between ETS and cancer. Mice exposed to ETS experimentally at high doses developed increased incidences of cancer (Hecht, 2005). Although most investigators continue to compare experimental results in animals with human data, most animals appear to be less sensitive than man

(Coggins, 2002). Beta-carotene supplementation attenuated cardiac remodeling induced by 1-month's tobacco smoke exposure in rats (Zornoff *et al.*, 2006). After 1-month's exposure to ETS, cardiac remodeling was present as indicated by disorganization or loss of myofilaments, in plasma membrane folding, dilatation of the sarcoplasmic reticulum and polymorphic mitochondria with swollen or decreased cristae. Rats given beta-carotene during cigarette smoke exposure had minimal or no significant cardiac remodeling. These data suggest beta-carotene attenuates, reduces or minimizes cardiac remodeling induced by cigarette smoke. These data are probably most relevant to obese cardiac-compromised companion animals. Differential diagnoses or conditions which must be adequately distinguished from smoke inhalation include asthma, heart disease, allergic pulmonary disease, inhalation of toxic solvents or sprays, pneumonia, neoplasia, trauma, chronic obstructive pulmonary disease (COPD) and pneumothorax (Fitzgerald and Vera, 2006).

Household use of metal oxide ENMs in sunscreens (mostly zinc oxide or titanium dioxide), or as cosmetics, has not led to reports of toxicities in humans (Nel *et al.*, 2006). Other metal oxide ENMs such as ferric oxide, however, are cytotoxic to mesothelioma cells and fibroblasts *in vitro* (Brunner *et al.*, 2006). In comparison, zinc oxide fumes at high doses (500 µg/m<sup>3</sup>), in an occupational setting, cause fume fever (fatigue, chills, fever, myalgia, cough and leukocytosis) (Nel *et al.*, 2006).

### Dog exposures to automobile exhaust aerosols

Repeated, high exposures to the complex pollutant mixtures in automobile exhaust aerosols cause lung structural changes induced by sustained inflammatory processes resulting in airway and vascular remodeling and altered repair. These exposures are complicated by a heavy load of oxidant gases. Bronchoalveolar lavage from dogs in heavily exhaust-polluted environments shows increased numbers of activated alveolar macrophages in association with ultrafine particles in alveolar type I and II cells. Cytokines released from both circulating inflammatory cells and resident lung cells, in response to endothelial and epithelial injury, are thought to play important roles. The lung changes are typically associated with myocardial and endothelial pathology (Calderon-Garciduenas *et al.*, 2001a, b).

## EMERGING CHALLENGES

A question that received recent scientific scrutiny is the potential indirect impact that ENMs may have as



contaminant carriers. For example, the uptake of phenanthrene by daphnids is increased by C<sub>60</sub> nanoparticles, and it increases phenanthrene toxicity to algae. Effects on uptake and toxicity are, however, variable depending on the organism and the toxicant (Baun *et al.*, 2008). The significance of these observations is still unclear, but it indicates that the possibility of ENMs affecting the potency and/or kinetics of toxicant co-exposures should be considered. On the other hand, ENMs can be used to reduce the impacts of environmental contaminants. Iron (0 valence/charge) particles, for example, may be used to remove chromium and lead in drinking water (Iseli *et al.*, 2009).

Evidence of ecotoxicological effects associated with nanomaterials is emerging. For example, leachates from low-purity fullerenes were shown to be toxic to aquatic organisms (Hull *et al.*, 2009). Nanotechnology may lead to novel types of pollutant effects. Organisms may not have the ability to metabolize and detoxify ENMs using pathways evolved to prevent poisoning from chemicals in solution. The diversity of environmental conditions and possible ecological impacts pose additional challenges to ecotoxicological risk assessment. Data from diverse test organisms are needed, and a general scarcity of data is still a major concern (Cattaneo *et al.*, 2009).

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# Ionizing radiation and radioactive materials in health and disease

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## INTRODUCTION

Ionizing radiation consists of electromagnetic waves or atomic particles with the capacity to strike an electron with sufficient force to strip it from its atom thus creating an ion. Ionizing radiation can also generate highly reactive nonionic species known as free radicals. However, ionizing radiation cannot be detected, even at lethal levels, without instruments designed for the purpose. Ionizing radiation is produced when nuclear weapons are detonated and when chest X-rays are taken. We are exposed to ionizing radiation all our lives and it may have beneficial or detrimental consequences. The objectives of this chapter are to describe common types of ionizing radiation from both natural and man-made sources, discuss how they are detected and measured, consider their uses especially in medical diagnosis and treatments, and provide information on both the possible health benefits and risks associated with ionizing radiation. All of the topics covered herein are treated more extensively in a concise, interesting, highly informative and well-written book ([Henriksen and Maillie, 2003](#)) that is highly recommended for those interested in a more in-depth look at this subject.

Roentgen, Henri Becquerel and Marie and Pierre Curie just before the turn of the 20th century. Wilhelm Roentgen discovered a form of penetrating radiation on November 8, 1895 when he observed that an electric discharge tube covered with black paper caused a dye outside the tube to fluoresce. Although the term Roentgen rays is used in many countries, the more common term for these electromagnetic waves is the name given to them by Roentgen himself, X-rays.

Henri Becquerel discovered radioactivity in the spring of 1896, when a photographic plate protected from visible light was exposed to a uranium salt. In July of 1898 Marie and Pierre Curie published their findings regarding the isolation of a new compound that they suggested be named polonium after Marie Curie's homeland of Poland. In this publication, they use the term radioactivity for the first time to describe the properties of the newly discovered element. A few months later, they published on another newly discovered radioactive element that they named radium. Marie Curie died in 1934 at the age of 67 from a blood disease, possibly leukemia, which may have been the result of her work with radioactive substances.

## TYPES OF IONIZING RADIATION

## HISTORICAL PERSPECTIVE

Our understanding of ionizing radiation began with the pioneering work of individuals like Wilhelm Conrad

The nucleus of an atom contains one or more protons and, with the exception of hydrogen, neutrons, while any associated electrons, frequently equal in number to the protons, move about the nucleus in specific orbitals.

TABLE 25.1 Naturally occurring radioactive families

Series	Half-life of parent (years)	Parent radioisotope	Stable decay product
Thorium	$1.41 \times 10^{10}$	Th-232	Pb-208
Uranium–radium	$4.47 \times 10^9$	U-238	Pb-206
Uranium–actinium	$7.04 \times 10^8$	U-235	Pb-207
Neptunium	$2.14 \times 10^6$	Np-237	Bi-209

Each element is defined by the number of protons in the nucleus, its atomic number ( $Z$ ),  $Z = 1$  for hydrogen,  $Z = 6$  for carbon,  $Z = 8$  for oxygen, etc. Although the number of protons defines the element, the number of neutrons defines the different isotope of that element. For example, hydrogen has no neutrons in its nucleus, but there are two additional isotopes of hydrogen, deuterium with one neutron and tritium which contains two neutrons. The atomic mass of an isotope is determined by the total number of nucleons (protons and neutrons). Electrons with a mass that is  $1/1838$ th that of a proton or neutron contributing little to the total atomic mass. The shorthand for hydrogen, deuterium and tritium, is  $^1\text{H}$ ,  $^2\text{H}$  and  $^3\text{H}$  (or H-1, H-2 and H-3), respectively. Most isotopes are stable, that is to say the number of protons and neutrons remains unchanged indefinitely, but some isotopes have unstable nuclei making them radioactive. For example, hydrogen and deuterium are stable isotopes while tritium is a radioactive isotope that is commonly used in biomedical research.

Radioactive isotopes go through a process of radioactive decay that involves emitting ionizing radiation in the form of subatomic particles and/or electromagnetic radiation. The source of radiation is the nucleus, and the decay continues until the nucleus is converted to a stable isotope. Tritium decays by emitting only one particle, becoming a stable isotope of helium (He-3 as opposed to the more common form of helium He-4), while uranium (U-238) emits a total of 14 particles before becoming a stable isotope of lead (Pb-206). There are four naturally occurring radioactive families on earth. Two of these have almost disappeared because their half-lives are relatively short in comparison to the age of the earth (Table 25.1) leaving only the thorium and the uranium–radium series.

Each decay event on the way to becoming a stable isotope is known as a disintegration and the rate of decay is given in disintegrations per minute (DPM) or disintegrations per second (becquerels abbreviated Bq). The particles emitted during radioactive decay include alpha and beta particles and neutrons. In addition to ionizing particles, radioactive decay can also produce a form of ionizing short wave electromagnetic radiation known as gamma rays. Important information about the steps involved in the decay of a radioactive isotope

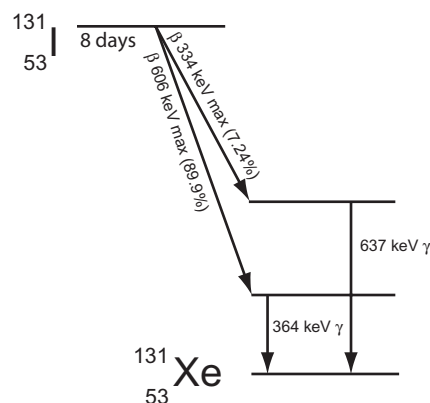


FIGURE 25.1 Decay scheme for radioactive I-131 showing beta particle emissions leading to the formation of a stable isotope of xenon (Xe-131). The decay occurs in two ways, 7.24% of the time a beta particle with maximum energy of 334 keV is emitted with the subsequent emission of a 637 keV gamma ray, and 89.9% of the time the decay occurs by the emission of a beta particle with a maximum energy of 606 keV and the subsequent emission of a 364 keV gamma ray.

is contained in its decay scheme. An example for iodine (I-131), an important radioisotope with regard to nuclear power plant accidents, is given in Figure 25.1.

Ionizing radiation can also originate from outside the atomic nucleus. Examples include X-rays and extreme ultraviolet radiation. Other atomic particles qualifying as ionizing radiation, but not considered here, include a variety of particles generated in particle accelerators and particles, other than alpha and beta particles, produced in nuclear fission and fusion reactions.

## Alpha and beta particles

Historically it was observed that radioisotopes emitted radiation that could be divided into three different types according to the way it behaved when subjected to a magnetic field. Two of them followed a curved path in a magnetic field, but in opposite directions indicating that they had opposite charges. The positively charged radiation was called alpha rays while the negatively charged radiation was referred to as beta rays. The third type of radiation was not deflected by a magnetic field and was referred to as gamma rays. In 1903, Ernest Rutherford discovered that alpha particles were equivalent to a helium nucleus consisting of two protons and two neutrons and having a charge of +2. Beta particles have the mass ( $9.1 \times 10^{-31}$  kg) and the negative charge ( $1.6 \times 10^{-19}$  coulombs) of an electron (see Table 25.2 for a list of units and magnitudes). Particles with the same mass and a charge opposite in sign but equal in magnitude to an electron are called positrons, and may also be referred to as beta particles. The kinetic energy imparted

TABLE 25.2 Abbreviations for units and orders of magnitude

Abbreviation	Parameter
<b>Units</b>	
Bq	becquerel
C	coulomb
erg	erg ( $10^{-7}$ J)
eV	electron volt
g	gram
Gy	gray
J	joule
m	meters
R	roentgen
rad	radiation absorbed dose
rem	roentgen equivalent in man (or mammals)
sec	seconds
Sv	sievert
<b>Magnitudes</b>	
a	atto ( $10^{-18}$ )
f	femto ( $10^{-15}$ )
p	pico ( $10^{-12}$ )
n	nano ( $10^{-9}$ )
$\mu$	micro ( $10^{-6}$ )
m	milli ( $10^{-3}$ )
c	centi ( $10^{-2}$ )
k	kilo ( $10^3$ )
M	mega or million ( $10^6$ )
G	giga ( $10^9$ )
T	tera ( $10^{12}$ )
P	peta ( $10^{15}$ )
E	exa ( $10^{18}$ )

to non-relativistic alpha and beta particles when they are ejected from the nucleus is given by the formula:

$$E = \frac{1}{2}mv^2$$

where  $E$  is the energy of the particle in joules,  $m$  is its mass in kg and  $v$  its velocity in m/sec. The unit of energy for this equation is the joule (J), but the energy of ionizing particles is usually given in electron volts (eV). One eV is the amount of energy imparted to an electron when it is accelerated by a potential difference of one volt. The factor for converting joules ( $\text{kg}\cdot\text{m}^2/\text{sec}^2$ ) to eV is:

$$1 \text{ eV} = 1 \text{ V} \times 1.6 \times 10^{-19} \text{ C} = 1.6 \times 10^{-19} \text{ J}$$

Ionizing particles have energies in the thousands (keV) or millions of electron volts (MeV):

$$1 \text{ KeV} = 1 \text{ eV} \times 10^3 \text{ and } 1 \text{ MeV} = 1 \text{ eV} \times 10^6$$

Alpha particles emitted by a particular radioisotope all have the same energy. For example, U-238 emits an alpha particle with an initial energy of 4.19 MeV, but there is variation in the energies of alpha particles from different isotopes. The energy of alpha particles ranges from 3 to 7 MeV while beta particles have energies

TABLE 25.3 Spectral ranges for X-rays and gamma rays

Radiation	Wavelength (nm)	Frequency ( $\text{sec}^{-1}$ or hertz)	Energy (keV)
Soft X-rays	10–0.1	$3 \times 10^{16}$ – $3 \times 10^{18}$	0.12–12
Hard X-rays	0.1–0.01	$3 \times 10^{18}$ – $3 \times 10^{19}$	12–120
Gamma rays, high energy X-rays	<0.01	$>3 \times 10^{19}$	>120

ranging from 20 keV to a few MeV. Beta particles from the same isotopic source vary in their energy. The value given in decay schemes represents the maximum energy (Figure 25.1). As a rule of thumb, the average energy is 1/3 of the maximum. The additional energy lost when lower energy beta particles are emitted is contained in a neutrino that is also emitted.

### Gamma rays

The wave length ( $\lambda$ ) of electromagnetic radiation varies from more than  $10^8$  meters in length to less than  $10^{-16}$  meters. The energy of electromagnetic waves is given by the equation:

$$E = hc/\lambda$$

where  $h$  is Planck's constant ( $6.626 \times 10^{-34} \text{ J}\cdot\text{s}$  or  $4.136 \times 10^{-15} \text{ eV}\cdot\text{s}$ ) and  $c$  is the speed of light ( $3.0 \times 10^8$  meters/sec). Only electromagnetic waves with  $\lambda$  of less than approximately  $10^{-8}$  meters have sufficient energy to qualify as ionizing radiation. Electromagnetic radiation with  $\lambda$  between  $1.2 \times 10^{-7}$  and  $10^{-8}$  meters are referred to as extreme ultraviolet (EUV) rays while X-rays generally range from  $10^{-8}$  to  $10^{-11}$  meters and gamma rays are any electromagnetic radiation with  $\lambda$  of less than  $10^{-11}$  meters (Table 25.3).

Plugging these values into the preceding equation give a range of energy of 0.01–0.12 keV for EUV, 0.12–120 keV for X-rays and  $>120$  keV for gamma rays.

Electromagnetic radiation interacts with matter in three ways: (1) the photoelectric effect, (2) Compton scattering and (3) pair formation. The photoelectric effect results in the complete absorption of a gamma photon's energy by an electron in the inner K and L shells of an atom. The electron escapes with energy ( $E_e$ ) approximately equal to that of the photon ( $E_\gamma$ ) less the binding energy of the electron ( $E_b$ ):

$$E_e = E_\gamma - E_b$$

$E_b$  varies with the atomic number ( $Z$ ) but is generally much less than  $E_\gamma$  so that the energy carried away by the electron is close to that of the incident gamma photon. Gamma photons can also be scattered by an encounter



with an electron. This is known as Compton scattering and results in the deflected gamma photon continuing in a new direction with reduced energy while the electron carries off the balance of the energy in another direction. Pair formation occurs when a gamma photon is converted into an electron–positron pair. The energy represented by an electron (or positron) with resting mass  $m_0$  ( $9.1 \times 10^{-31}$  kg) is given by  $m_0c^2$ . Since  $c = 3 \times 10^8$  m/sec and  $1 \text{ MeV} = 1.6 \times 10^{-13}$  J ( $\text{kg}\cdot\text{m}^2/\text{sec}^2$ ):

$$m_0c^2 = 0.51 \text{ MeV}$$

Because both an electron and a positron are formed the photon's energy must be at least 1.02 MeV. Any energy in excess of this amount is shared by the particles as kinetic energy. Positrons are unstable and after coming to rest will quickly recombine with resting electrons producing a pair of gamma photons each of 0.51 MeV and moving in opposite directions.

## X-rays

When a beam of electrons hits a heavy metal target, their rapid deceleration by positively charged atomic nuclei within the target, causes some of the electrons energy to be converted to electromagnetic radiation in the form of X-rays. Radiation produced in this way is also known as bremsstrahlung. The beam of electrons originates at the negatively charged cathode within an evacuated tube and is accelerated toward the metal target (the anode) by a voltage difference between the two electrodes. The energy of the X-rays depends upon the magnitude of this voltage difference. X-rays generated by voltage differences of 100 to 300 kV have enough energy to ionize molecules in the target tissue by Compton scattering. The electrons that are stripped from molecules are known as secondary electrons, and if they have sufficient energy to produce additional ionizations, then they are known as delta rays. X-rays produced with high voltages have important diagnostic and therapeutic applications. The energy of X-rays produced by X-ray tubes is limited by the potential difference that can be applied to the electrodes. The practical limit for conventional X-ray tubes is 300 kV. By definition the electrons produced by this voltage would have a maximum energy of 300 keV and are capable of producing X-rays with the same maximum energy. Only about 1% of the electrons striking the anode generate X-rays, and their average energy is 1/3 of the maximum. The energy of the remainder of the electrons is converted to heat. Generating X-rays with energies on the order of 10 MeV requires a linear accelerator. An X-ray with energy of 10 MeV would correspond to a wavelength on the order of  $10^{-13}$  m or 0.0001 nm. This overlaps extensively with

gamma rays (Table 25.3). What distinguishes gamma rays from X-rays is not wavelength but the source of the radiation. Gamma rays originate in the nucleus of an atom while X-rays arise from sources outside the nucleus.

X-ray tube voltages between 25 and 50 kV are used to generate “soft” X-rays (Table 25.3) which are absorbed completely after producing a single ionization by the photoelectric effect described above. Soft X-rays are important for medical diagnostic procedures such as mammography because they produce better contrast between tissues that do not differ greatly in electron density making it easier to distinguish between tumor tissue and normal tissue.

## MEASURING IONIZING RADIATION

Almost everyone has experienced a sun burn and understands that there is a correlation between the dose of ultraviolet radiation received from the sun and skin redness. Originally, skin reddening following exposure to ionizing radiation was used to measure the absorbed dose and was known as the skin erythema dose. There are obvious drawbacks to this method of measuring dose. Redness caused by a given dose is going to vary with the individual making it a qualitative rather than a quantitative measure of exposure. There is a delay before the dose can be assessed and the method also lacks sensitivity. The erythema dose was replaced by the roentgen (R) unit which is equivalent to the amount of X-rays or gamma rays that generate  $2.58 \times 10^{-4}$  coulombs of ions/kg of air. In 1953, the radiation absorbed dose (rad) was adopted which is equal to the amount of radiation which yields an energy absorption of 100 erg/g (i.e.  $10^{-2}$  joules/kg). Although the rad is still commonly used, it is being replaced by the Système International (SI) unit known as the gray (Gy), named after the radiation biologist L. Harold Gray. The relationship between R, rad and Gy is given in Table 25.4.

The amount of energy deposited as ionizing radiation travels through tissue is known as the linear energy transfer (LET) and is given in  $\text{keV}\cdot\mu\text{m}^{-1}$ . Alpha particles do not travel very far, only a few centimeters in air and less than a millimeter in water or soft tissue. They are stopped by clothing or the first few cells of the skin but the LET for these particles is very high compared with other forms of radiation and most of the energy is deposited near the end of its track producing a peak called the Bragg peak (Figure 25.2).

By comparison, beta particles of 1 MeV will penetrate to about 0.5 centimeters in water or soft tissue, and have a much lower LET. Damage to tissue is related to LET.

TABLE 25.4 Units used to measure the activity of a source (Bq) and radiation doses (Gy and Sv)

SI unit	Dimensions	Other units	Equivalencies
Becquerel (Bq)	Disintegrations/sec (DPS)	Curie (Ci)	$37 \times 10^9 \text{ Bq} = 1 \text{ Ci}$
Gray (Gy)	J/kg	Roentgen (R) = $2.58 \times 10^{-4} \text{ Ci/kg ions}$	$1 \text{ Gy} \approx 108 \text{ R}$ (for gamma rays in soft tissue)
		Radiation absorbed dose (rad) = $100 \text{ erg/g}$	$1 \text{ Gy} = 100 \text{ rad}$
Sievert (Sv)	J/kg	Roentgen equivalent in mammals (rem)	$1 \text{ Sv} = 100 \text{ rem}$

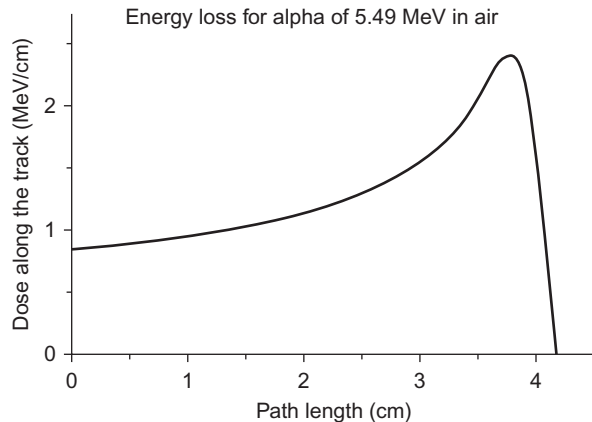


FIGURE 25.2 Bragg curve for the deposition of energy by an alpha particle along its track in air.

Absorbed dose in Gy does not take into account disparities in the ability of different types of radiation to cause tissue damage. Therefore another unit of measure known as the equivalent dose was established. Originally, the units were known as roentgen equivalent in man (or mammals), abbreviated rem. The SI unit for equivalent dose is the sievert (Sv) named after the Swedish scientist Rolf M. Sievert, and its relationship to the absorbed dose is given by the equation:

$$\text{Sv} = w_R \cdot \text{Gy}$$

where  $w_R$  is the radiation weighting factor for a particular type of radiation, and is proportional to its LET. The relationship between rem and Sv is given in Table 25.4. X-rays and gamma rays have a low LET depositing energy at a low rate along their track often emerging from the body and continuing on their way. This is the reason why X-rays can be used to generate images on a photographic plate. X-rays pass through soft tissue and reach the film placed opposite that part of the body being X-rayed. When the film is developed, areas that were exposed to X-rays appear dark. Dense tissues such as bone absorb most of the X-rays producing, in essence, a shadow on the film that is revealed by lighter areas on the developed film. Since the LET for X-rays, as well as gamma rays, is the lowest for ionizing radiation their  $w_R$  value is set at one. In contrast, the alpha particles emitted by a source that is in direct contact with tissue

TABLE 25.5 Weighting factors for various types of ionizing radiation used for converting absorbed dose in Gy to equivalent dose in Sv

Type of ionizing radiation	$w_R$
X-rays, gamma rays, beta particles	1
Protons >2 MeV	2
Neutrons	5
<10 keV	
10 keV–100 keV	10
100 keV–2 MeV	20
2 MeV–20 MeV	10
>20 MeV	5
Alpha particles, nuclear fission products	20

deposit all of their energy in the tissue over the space of a few millimeters, hence they have a high LET and are assigned a  $w_R$  value of 20. The values of  $w_R$  have been arrived at experimentally but are nonetheless difficult to determine quantitatively, which is one reason why accessing the equivalent dose can be difficult. Values of  $w_R$  are given in Table 25.5. Complicating matters is the fact that different organs vary in their sensitivity to the effects of radiation. This is taken into account by using a tissue weighting factor which varies for different organs. The weighting factor is highest for more sensitive organs and the sum of the weighting factors for all organs is 1.0. Gonads are considered the most sensitive and are given a weighting factor of 0.20, while skin ranks at the bottom with a weighting factor of 0.01 (Henriksen and Maillie, 2003). Multiplying the weighting factor by the equivalent dose gives what is known as the effective equivalent dose for a given organ. In the case of whole body irradiation, the weighting factor is, of course, 1.0, so equivalent dose is equal to the effective equivalent dose.

## DEVICES FOR MEASURING IONIZING RADIATION

Devices used for detecting and measuring the amount (Bq) and energy (MeV) of radiation and for estimates of the absorbed or equivalent dose (Gy or Sv) take advantage of the ability of radiation to cause ionization. These devices are treated summarily here. Much more detail can be found elsewhere (Delaney and Finch, 1992) for

those who are interested. These devices can be divided into two categories; those that store information about radiation exposure with the information requiring retrieval at some later time, and those that are able to detect radiation as it occurs.

## Film

Photographic film is sensitive to ionizing radiation just as it is to light. The film emulsion contains crystals of silver halide, usually silver bromide. In essence, ionizing radiation facilitates the transfer of electrons from the bromide ions to silver ions producing grains of elemental silver. With increasing exposure, the film becomes progressively more opaque as more and more silver grains are deposited until the film finally appears black when developed. Thus, the amount of silver deposited is related to the dose of radiation received. The film is inserted into a film badge holder and clipped to a worker's clothes serving as a personal dosimeter. Portions of the film can be covered with different materials in order to gain information about the type of exposure an individual receives. For example, film behind a cadmium plate will be more highly exposed than the unmasked portion of the film if the dosimeter is exposed to low energy neutrons. Filters may also be used on part of the film to distinguish between exposure to beta and gamma radiation.

## Thermoluminescent dosimeter

Thermal luminescence dosimeters (TLD) are composed of lithium fluoride (LiF) crystals. When these crystals are exposed to radiation, electrons become trapped in the excited state. When the crystal is heated to approximately 200°C these electrons give up their excess energy emitting photons in proportion to the amount of radiation absorbed. TLD make good dosimeters because they mimic the properties of tissue with regard to absorption of radiation. They have advantages over film in that no film development is required and they can be reused many times. The disadvantages are that the information stored in the TLD can only be retrieved once, and TLDs do not reveal as much information about the types of radiation exposure as film can.

## Gas-filled detectors

Pulse ionization detectors have a gas-filled chamber containing two electrodes to which a voltage is applied in order to generate an electrical field. Ionizing radiation enters the chamber through a window in the detector and ionizes gas within the detector, typically a mixture of 90% argon and 10% methane. The electrons from the

ionization are attracted to the anode while the positively charged cations are drawn to the negatively charged cathode. Arrival of these ions at the electrodes causes a voltage change that can be detected. Since the energy of an individual particle or photon determines how many ionizations it can cause, the number of ions arriving at the electrodes, and hence the voltage change, will be proportional to the energy of the radiation being detected. The energy spectrum reveals information about the type of radiation and can be used to identify the source. However, an accurate determination of the energy of a particle or photon depends upon all of its energy being deposited in the gas within the chamber. This is not a problem with alpha particles which travel only a few centimeters in a gas before giving up all of their energy. In this case, the primary concern is admitting the alpha particles through the detector window. Windowless detectors exist which require that a flow of gas be maintained in order to preserve the environment between the electrodes. In contrast, high energy beta particles and gamma radiation will emerge from a detector of reasonable dimension before they have given up all of their energy so that an accurate energy spectrum cannot be obtained.

If the voltage applied to the electrodes is increased, the ions produced by the radiation entering the detector will be accelerated toward the electrodes, giving them enough energy to ionize additional molecules and atoms. There is a range of voltage in which the increased number of ions produced is proportional to the energy of the particle of ionizing radiation. Detectors known as proportional counters operate in this voltage range. These detectors have higher sensitivity than the pulse ionization detectors discussed at the outset of this section. The time required to register the voltage change associated with a single particle is on the order of a few  $\mu\text{sec}$ . Thus, individual events can be resolved even at a relatively high rate of incidence allowing a determination of the number of decays per unit time for a particular source.

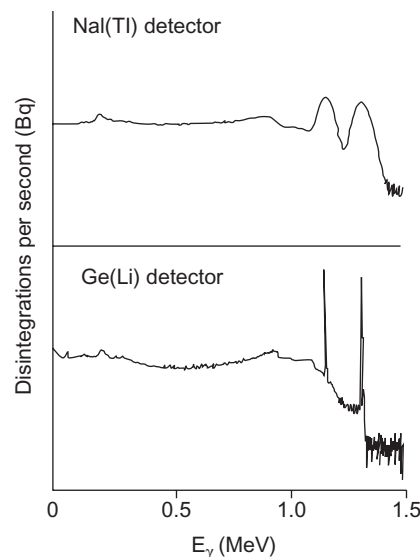
If the electrode voltage is increased above the proportional range, the ionizing radiation initiates a cascade of ionizations, and the resulting voltage change recorded by the instrument is independent of the original particle's energy. These electrode voltages are in the Geiger-Muller range and are used in Geiger counters. The advantages of these instruments are that they are inexpensive to manufacture and sensitive, which makes it easy to detect low energy radiation. A disadvantage is that they do not give any information about the energy of the particles being measured and their design prevents them from being able to accurately measure dose rates much in excess of 50 Bq. As with other gas-filled detectors, Geiger counters are capable of detecting low to moderate energy beta particles and some alpha particles but are not very useful for high energy beta particles and can only detect 1–2% of incident gamma radiation.

## Scintillation counters

Scintillators are materials that emit light (usually ranging from visible blue to near ultraviolet) in response to ionizing radiation. Scintillation counts have a photomultiplier tube that generates an electrical pulse which is proportional to the amount of light produced by the scintillator. Scintillators can be organic compounds such as molecules containing benzene rings or inorganic compounds such as crystals of sodium iodide containing traces of thallium – otherwise written as NaI(Tl). Crystals on account of their density are ideal for stopping gamma and X-rays, forcing them to give up all of their energy to the crystal. Organic scintillators can be dissolved in liquids which also have good X-ray and gamma ray stopping power. In scintillation counting the ions produced by the incident radiation subsequently excite electrons associated with the scintillator. Light is produced when these excited electrons return to the ground state. The number of scintillators that are excited, and consequently the amount of light emitted, is proportional to the energy of the ions produced by the radiation. As discussed above, electromagnetic waves interact with matter in several different ways, one of these being by the photoelectric effect. Since the photoelectric effect generates a photoelectron whose energy is nearly equal to that of the original photon, the amount of light produced when the photoelectron excites the scintillator is a function of the original photon's energy and produces what is known as the photopeak in the energy spectrum. The photopeak is characteristic of the emitter making it feasible to identify the isotope that produced it. An example is shown in Figure 25.3. Compton scattering also contributes to the energy spectrum and is broadly distributed as energies up to two thirds that of the photopeak. The reason is that the electrons produced by Compton scattering are of varying and lesser energy compared with the photoelectrons. The contribution of Compton scattering can also be seen in Figure 25.3. Beta particles also produce photopeaks that reveal information about their energy. Depending on the scintillator, alpha particles produce between 10 and 100% of the light that is produced by an electron or beta particle with equivalent energy. In addition to measuring the energy of ionizing radiation, scintillation counters measure the event rate with resolution in the  $\mu\text{sec}$  range making it possible to accurately measure the Bq for sources with relatively high rates of decay.

## Semiconductor detectors

When a voltage is applied to a pair of electrodes bridged by an ideal semiconductor no current flow is induced by the electrical field. Ionizing radiation can transfer energy to electrons within the semiconductor making



**FIGURE 25.3** Energy spectrum for Co-60 determined with an NaI(Tl) scintillation detector and with a germanium semiconductor detector. Two photopeaks are observed corresponding to gamma radiation with energies of 1.17 and 1.33 MeV.

them susceptible to the electric field so that they drift in the direction of the anode while the electron's counterpart, referred to as a hole, behaves like a positively charged ion and drifts in the opposite direction toward the cathode. This movement of ions induces a current that can be measured. The principle of operation is similar to that of gas-filled detectors, and semiconductor detectors produce analogous energy spectra useful for fingerprinting the source. Of significance is the fact that in gas-filled detectors and scintillators, approximately 25 and 100 eV are required, respectively, to produce a single ionization, whereas in operational semiconductor detectors only 3 eV is needed to produce an electron-hole pair. This disparity means that semiconductor detectors produce energy spectra with higher resolution than do gas-filled detectors or scintillation counters as can be seen from looking at Figure 25.3. The reason for this is that an ionizing particle will produce many more electron-hole pairs in a semiconductor than it does ion pairs in a gas-filled detector or scintillation counter, thus reducing the statistical variation for semiconductors compared with the other types of detectors. Another advantage of semiconductor detectors is that the stopping time for ionizing radiation in these detectors is very short, and consequently the period during which detection takes place is correspondingly short. As a result, the time resolution of these detectors is in the nanosecond range enabling these detectors to record very high dose rates. A disadvantage of semiconductor detectors, which are made of silicone or germanium, is that they are not ideal semiconductors. When an electrical field is applied to these detectors, a



current flow is created that constitutes a background of sufficient magnitude to obscure any signal produced by ionizing radiation. The solution is to reduce the background by cooling the detector to  $-196^{\circ}\text{C}$  with liquid nitrogen.

## IONIZING RADIATION: RISKS AND BENEFITS

Exposure to ionizing radiation from natural sources comes from radioisotopes found in the earth's crust and oceans, cosmic radiation from outer space and internal sources deposited when naturally occurring radioactive materials are inhaled or ingested. Exposure from these sources constitutes the total dose that most life on earth receives, and approximately half the annual dose to humans. The other half of what humans receive comes primarily from medical diagnostic tests and therapeutic applications of radiation used for curative or palliative procedures. Some populations of humans and other species have been exposed to radiation from nuclear bomb blasts during war time and during tests conducted in peace time, and as a consequence of nuclear power plant accidents.

Radiation exposure carries with it both risk and benefit, and although the objective is to maximize benefit while minimizing risk, this is not necessarily easy to achieve. For example, a medical diagnostic procedure such as mammography requires exposure to radiation, but can be essential for catching breast cancer in its early stages when it can be effectively treated and a cure more likely achieved. On the other hand, radiation exposure at high enough levels increases the risk of developing latent effects such as cancer that appear months or years later. Although an annual mammogram does not result in a dose of radiation that is associated with an increased risk of developing cancer, multiple exposures of the chest area as a consequence of several diagnostic procedures prescribed during the course of a year could measurably increase the risk of developing latent effects. Following the bombings of Hiroshima and Nagasaki the incidence of leukemia did not peak for 5–7 years, and following the Chernobyl disaster the appearance of thyroid cancer reached a maximum 6–7 years after the accident. Generally, lower doses of radiation are associated with longer latency in the appearance of latent effects.

### Mechanisms of injury and their consequences

The minimum energy needed for a single ionization event is 6–10 eV. Thus 1 MeV of radiation has the

potential to produce about 150,000 ionizations. As discussed, above electromagnetic radiations, including gamma rays and X-rays, have a relatively low LET, hence the reason for assigning them a  $w_R$  value of one. The penetration of electromagnetic radiation is given by the formula:

$$I(x) = I_0 e^{-\mu x}$$

where  $I_0$  and  $I(x)$  are the initial intensity and intensity at a distance  $x$  into the material, and  $\mu$  is the absorption coefficient of the material. The three types of interactions for electromagnetic radiation with matter described above contribute to  $\mu$  so that it can be rewritten as:

$$\mu = \mu_{\text{PE}} + \mu_{\text{C}} + \mu_{\text{P}}$$

where  $\mu_{\text{PE}}$ ,  $\mu_{\text{C}}$  and  $\mu_{\text{P}}$  are the respective contributions to the absorption coefficient by the photoelectric effect, Compton scattering and pair production. A derivation of the quantitative expression for each of these components is beyond the scope of this chapter, but the following relationships are informative:

$$\mu_{\text{PE}} \propto N(Z^5/E_{\gamma}^3)$$

where  $N$  is the number of absorber atoms per unit volume,  $Z$  is the atomic number of the absorber and  $E_{\gamma}$  is the energy of the radiation. Absorption due to the photoelectric effect increases sharply with atomic number making it an important factor in absorption by tissues like calcium-laden bone. There is also a precipitous decrease in absorption due to the photoelectric effect as energy of the radiation increases, indicating that the photoelectric effect is important in the absorption of lower energy radiation. The contribution of Compton scattering to the absorption coefficient is given by the proportionality:

$$\mu_{\text{C}} \propto NZ/E_{\gamma}$$

As with the photoelectric effect, absorption by Compton scattering varies directly with the atomic number of the absorber and inversely with the energy of the radiation. In contrast to the photoelectric effect, the relationships are linear not exponential. Consequently, scattered photons are more likely to escape absorption so as not to deposit all of their energy in the tissue. Absorption due to pair production is given by the approximation:

$$\mu_{\text{P}} \cong NZ^2(E_{\gamma} - 2m_0c^2)$$

Obviously absorption by pair production does not occur until  $E_{\gamma} = 2m_0c^2$  (1.02 MeV). For radiation with energy in excess of 1.02 MeV, absorption by this mechanism becomes important.

A radioactive substance that emits gamma rays is easy to detect if ingested because, as noted above, the radiation is most often not entirely absorbed by the body and can be detected externally with instruments. In contrast, radioactive materials such as Sr-90 and Pu-239, isotopes that would be released in a nuclear power plant accident or by the detonation of nuclear weapons, emit only alpha and beta particles. They have a high LET and deposit all of their energy within millimeters or centimeters of their source. Consequently, if inhaled or ingested they do not escape the body making them difficult to detect. When evaluating the health effects of ingested or inhaled radioisotopes, the biological half-life ( $t_b$ ), the time needed to excrete half the radioactive material from the body, is an important consideration. Values of  $t_b$  vary with species, age and the isotope taken in. For example, Cs-137 released in the Chernobyl accident was found in the bodies of humans and sheep. Even though it has a  $t_{1/2}$  of 30 years, its  $t_b$  was 3 months in humans and only 2–3 weeks in sheep. Since the  $t_b$  is relatively short in sheep, it is not unreasonable to “feed down” the animals before slaughter to bring the radioactive content of the meat to a level that does not present a health risk when consumed. Including some agents such as Berlin blue in the feed can also reduce the  $t_b$ . Using non-radioactive isotopes to compete with a radioisotope can also be effective in reducing the  $t_b$  or as a prophylaxis in advance of a potential exposure. For example, taking non-radioactive I-130 will compete with the radioisotope I-131 for uptake by the thyroid, thus protecting it from damage by the latter. For radioisotopes such as radium and strontium, which are deposited in bone and remain there indefinitely, the  $t_b$  is equal to the  $t_{1/2}$ . Radioisotopes also have what is known as a radio-ecological  $t_{1/2}$  defined as the half-life for the animals and plants living in a particular area. Radio-ecological  $t_{1/2}$  may be difficult to determine and the disappearance of radioactivity from the environment may not follow an exponential decay curve classically described by a half-life equation.

Death due to radiation that occurs within 30 days of exposure is known as acute radiation death. The doses that will cause acute radiation death in half of the exposed population (the LD<sub>50</sub>) for different species are given in Table 25.6 (Henriksen and Maillie, 2003). The pathological effects of radiation depend upon the dose that is received. Rapidly growing cells, such as cancer cells, intestinal epithelial cells and hematopoietic cells, are more susceptible to high doses of radiation than are quiescent cells. For humans, a dose of 3–10 Gy causes hematopoietic syndrome, which results from damage to the bone marrow and destroys the body’s ability to produce erythrocytes and cells of the immune system. The victim experiences nausea, vomiting, and diarrhea, and without the ability to fight infection, death occurs within a few weeks. Gastrointestinal syndrome results from a

TABLE 25.6 Acute doses of radiation that result in 50% lethality (LD<sub>50</sub>) of an exposed population

Organism	LD <sub>50</sub> dose in Gy
Human	3–5
Dog	3.5
Monkey	6
Frog	7
Rat	7.5
Rabbit	8
Tortoise	15
Goldfish	23
Bacteria	>1000

dose of 10–100 Gy in which the cells of the intestinal epithelium are destroyed as is the ability to produce blood cells. The victim experiences stomach and intestinal pain, nausea, vomiting and diarrhea with death occurring within 3–10 days in dogs (Hanford, 1960) and humans. Doses greater than 100 Gy cause central nervous system syndrome that manifests as irritability, hyperactive response, fatigue, vomiting, diarrhea, loss of coordination, shivering, coma and death within 48 hours.

The greatest number of war-related acute radiation syndrome deaths occurred in the bombing of Hiroshima and Nagasaki in 1945, and the majority of acute radiation syndrome deaths caused by accidents occurred at Chernobyl. There is only one confirmed case in which a radioactive material has been used successfully to assassinate someone. Alexander Litvinenko, a former officer in the Russian Federal Security Service, escaped prosecution in Russia and received political asylum in the United Kingdom. As the author of *Blowing up Russia: Terror from Within* and *Lubyanka Criminal Group*, he accused the Russian secret service of carrying out acts of terrorism to bring Vladimir Putin to power. On November 1, 2006, Litvinenko suddenly fell ill and was hospitalized. He died 3 weeks later, the victim of lethal polonium-210-induced acute radiation syndrome. This set off an investigation that led to a diplomatic rift between the British and Russian governments. British officials had identified a former officer of the Russian Federal Protective Services, Andrei Lugovoy, as the primary suspect. Unsuccessful attempts were made to have him extradited to Britain and subsequent election to the Duma secured his immunity from prosecution.

By and large, acute radiation syndrome does not represent a serious health issue simply because it so rarely happens and is generally preventable. On the other hand, latent effects caused by much lower doses of radiation than those responsible for acute radiation syndrome need to be considered with regard to health policies. Any of the macromolecules that go into the making of a cell including proteins that are encoded by approximately 30,000 human genes together with the lipids and

carbohydrates that these proteins are often responsible for metabolizing can be damaged by radiation. At the high doses of radiation that cause acute radiation death, the damage is so severe that the cell cannot recover, and consequently dies as a result of the initial insult or because the cell becomes apoptotic which in essence, means that it commits suicide. There are hundreds or thousands or millions of copies of each of these macromolecules, and as long as the genes that encode the proteins are not irreparably damaged, new proteins can be made to replace those that are no longer functional. If the dose of radiation is less than 4–5 Gy, it is likely that the most sensitive cells in bone marrow and the gastrointestinal tract will recover and the victim will live. In these cases, it is latent effects, primarily the appearance of cancer, that become a health issue. The event that gives rise to these effects is usually damage to the genetic material, the DNA, of the cell. DNA is made up of two helical strands each consisting of a backbone of alternating sugar and phosphate molecules with purine (adenine or guanine) or pyrimidine (cytosine or thymine) bases attached to the sugar moieties. The bases on each strand form non-covalent-bonded base pairs with each other (guanine paired with cytosine and adenine paired with thymine) that knit the two DNA backbones together. Unlike the other macromolecules in the cell, there are most often only two copies of the gene that encode a particular protein. Many proteins are enzymes that function as catalysts in metabolic pathways within the cell. Some of these are enzymes that ensure the fidelity of DNA replication and repair damaged DNA. Endonucleases and exonucleases remove mismatched or damaged bases, polymerases replace them and ligases ensure that there are no nicks in the DNA backbone. Damage occurs when radiation causes breaks in the DNA. A break in the backbone of one strand is quickly repaired. Multiple breaks can also occur sometimes in close proximity and on opposite DNA strands. Such breaks are associated with cell death in prokaryotic bacteria. In eukaryotes, the damage can be repaired, but not as easily as a single strand break. If genes that encode DNA repair enzymes are damaged and the damage is not repaired the result could be mutations that alter or destroy the activity of the enzymes. If the ability of the cell to repair DNA damage is compromised, additional mutations may occur. Furthermore, there are proteins that act to stop the uncontrolled growth of a cell by triggering apoptosis and killing the cell rather than allowing it to become cancerous. In cells lacking an intact DNA repair machinery, damage of the DNA that codes for these critical proteins cannot be effectively repaired and the encoded proteins may not be functional, resulting in an increased risk of the cell becoming cancerous. Consider the following example. Ultraviolet radiation can cause the covalent linkage of adjacent pyrimidine bases (cytosine or

thymine) generating pyrimidine dimers. Normally the damage can be repaired by the cell, but individuals with a genetic disorder known as xeroderma pigmentosum have mutations in some of the DNA repair proteins and enzymes that compromise or destroy their activity. These individuals are predisposed to developing skin cancer that is often lethal.

Radiation damage to DNA or more correctly to chromatin (a complex of DNA and proteins) can be observed at the microscopic level. Cells go through a series of phases known as the cell cycle in the process of dividing. During metaphase, the chromatin is compacted so that the chromosomes can be seen under a light microscope. The chromosome consists of a pair of chromatids that are equal in length and joined by a single centromere in such a way that each chromatid has a long arm and a short arm. Radiation can generate chromosomal aberrations in the form of rings (ring formation) or the presence of two centromeres (dicentric). The percentage of cells with chromosomal aberrations increases with the dose of radiation, and can be used as a biological dosimeter for exposures of 0.2 to 12 Gy.

## Natural sources

Cosmic radiation and naturally occurring radioactive materials in the earth's atmosphere, the earth's crust and in its oceans constitute the natural sources of radiation that all organisms are exposed to. About 85–89% of incoming cosmic ray nuclei are protons (hydrogen nuclei), 10–13% are alpha particles and 1% of cosmic ray nuclei are those of the heavier elements. These nuclei together make up 99% of cosmic rays, and electrons (which cannot be distinguished from beta particles because their source, whether it be atomic nuclei or extra nuclear, is not known) constitute the remaining 1% of the particles that make up galactic cosmic rays. Earlier, it was stated that alpha particles with energies in the range of 3–7 MeV only travel a few centimeters in air, so how is it that they can reach the earth's surface from outer space? The reason is that the particles in cosmic rays reach the earth with energies on the order of  $10^{14}$  MeV. The earth's atmosphere acts as a partial shield against cosmic rays, and as you travel away from the earth's surface, for example when flying on an airplane or living at higher elevations, there is an increase in the absorbed dose during that period of time. On a typical airplane flight, the dose rate from cosmic radiation at cruising altitude is approximately 40 to 100 times that at sea level.

The other source of natural radiation comes from radioisotopes that were incorporated into the earth's crust when it was formed. The most important elements in this regard are uranium-238 and thorium-232 and the radioisotopes that result from their decay (their radioactive

**TABLE 25.7** Typical doses of radiation received from natural environmental sources

Source	Annual dose in mSv
Cosmic radiation (airline crew)	3–5
Cosmic radiation (exposure at sea level)	0.3–0.6
Natural gamma radiation	0.5
Buildings	0.9–1.3
Food	0.3–0.4

families), and potassium-40. These radioisotopes are not evenly distributed over the earth's surface. Consequently, the dose of radiation received will vary depending upon locality. Being inside a building does not necessarily offer protection from radiation because the construction materials used in making the building will contain radioisotopes. The use of shale in the construction of homes in Sweden was banned in 1979 because it had unacceptably high levels of uranium. Homes made of red brick often have higher levels of radiation contributed by K-40. Depending upon the construction materials and the place of their origin, the dose received from a building can vary by a factor of about two.

Exposure to radiation also comes from food with the principal contributor being K-40 and C-14. K-40 with a  $t_{1/2}$  of  $1.25 \times 10^9$  years accounts for about half of the dose due to internal sources, contributing 40–70 Bq/kg body weight depending upon sex and age. Carbon makes up the other half contributing about 50 Bq/kg and there are traces of 3-H that contribute less than 1 Bq/kg. A summary of the contribution that various natural sources give to the total annual absorbed dose is provided in Table 25.7. It is enlightening to realize that the average background exposure to natural radiation causes approximately  $5 \times 10^8$  ionizations per second in humans with average body mass. The rate is proportional to the target size so, if you happen to be a sumo wrestler your body may be subjected to a billion ionizations per second.

## Nuclear weapons

There have been more than 2000 nuclear weapons detonated since 1945 and 461 of these have been in the atmosphere. Most atmospheric testing ceased in 1963 with the exception of France where atmospheric tests continued until 1974 and China where they continued until 1980. Explosions near ground level draw dirt and debris into the mushroom cloud creating large amounts of radioactive fallout. Despite atmospheric testing, the radiation received by the average individual worldwide is not increased measurably over background, and barring future tests or the use of nuclear weapons in war this will not change. Obviously, there are populations

that have received high doses of radiation from nuclear explosions. The combined death toll in Hiroshima and Nagasaki was estimated to be 103,000. The majority, approximately 67,000, were killed in the initial blast and 36,000 in the 4 months following the attacks. It is not known how many in the latter group died as the result of acute radiation syndrome. The estimated average dose of radiation to those that were exposed was 160 mSv. During nuclear tests, efforts are made to protect humans from exposure to radiation, but a test detonation by the United States in the Bikini atoll in 1954 exposed the crew of 23 men aboard the Japanese tuna fishing boat *Daigo Fukury Maru* to an estimated 2–6 Sv of radiation. When examined in 1982, these men had an increased number of chromosomal aberrations in their lymphocytes as did survivors of the nuclear bombs dropped on Hiroshima and Nagasaki.

## Nuclear power

Normally, there is negligible release of radioactive materials from a properly operating nuclear power plant. Releases that are of concern occur during accidents. In October of 1957, a fire at the reactor at Windscale in the UK led to the release of an estimated 600 TBq of I-131, 45 TBq of Cs-137 and 0.2 TBq of Sr-90 (Crick and Linsley, 1984). On March 28, 1979, the reactor at the power plant on Three Mile Island near Harrisburg, Pennsylvania, experienced a partial meltdown before the situation could be brought under control. Only small amounts of radioactivity were released. On April 26, 1986, there was a catastrophic accident at the Chernobyl power plant in Ukraine. There were 31 deaths caused by the accident, 28 of which were attributed to acute radiation syndrome. An estimated 260,000 TBq of I-131, 38,000 TBq of Cs-137 and 8000 TBq of Sr-90 were released along with a large amount of other radioactive materials. Cs-137 and Sr-90 are of concern because of their relatively long  $t_{1/2}$  of 30.0 and 29.2 years, respectively. Although I-131 has a short  $t_{1/2}$  of just over 8 days, it is concentrated by the thyroid, increasing the likelihood of acute tissue damage or latent effects. This is exemplified by the fact that 14 years after the Chernobyl accident the only prevalent disease was thyroid cancer in children with about 700 cases being reported.

At 2:46 pm local time on March 11, 2011, a magnitude 8.9 earthquake centered just off the northeast coast of Japan generated a tsunami that came ashore moments later. The earthquake and ensuing tsunami damaged the Fukushima Dai-ichi nuclear power plant and disrupted the power needed to cool the reactor. The situation is ongoing and the level on the International Nuclear and Radiological Event Scale (INES) established in 1990 by the International Atomic Energy Agency (IAEA) remains



at 7 – the highest level. Thus far, there have been no casualties though some workers were exposed to radiation and the accident led to the evacuation of residents within a 20 km radius of the plant. It has been estimated that the release of radioactivity is about 1/10th of what occurred at Chernobyl.

### Sterilization of food products

Radiation has been used since the 1950s to sterilize food products. A source of gamma rays such as Co-60 or Cs-137 is used for the purpose though ultraviolet radiation is preferred for some foods, milk being an example, because its flavor is changed by gamma rays. Typically, the dose of gamma rays is 5–10 kGy and there is no contamination of the food by the radioactive source because it never comes into contact with the food. Keep in mind that the food itself will naturally contain small amounts of radioactive materials, primarily K-40 and C-14. The use of radiation is an effective way of destroying pathogens like *Salmonella* bacteria. As has been discussed, ionizing radiation by its nature has the ability to cause chemical changes in the materials that it comes into contact with and the fact that the flavor of milk is altered by gamma rays attests to this fact. However, studies have shown that the doses of radiation used for treating foods causes minor chemical changes that are not harmful to humans. It is appropriate to point out that other methods of food preservation including heating and the addition of chemical preservatives also cause chemical changes in the food. One advantage of using radiation for food preservation is that it often does a superior job of protecting food quality compared with other methods of sterilization.

### Medical diagnostics and treatments

Radiation has been used for more than 50 years to sterilize certain medical equipment and supplies, bandages for example, that are not amenable to sterilization by high temperature steam in an autoclave. Sterilization is achieved by exposure to gamma rays from a Co-60 source that delivers doses of 20–40 kGy. Using radiation to sterilize equipment and supplies does not expose patients or health care workers to radiation unless there is an accident like the one that occurred in Norway in September of 1982. The failure of a safety lock and warning system coupled with a failure to follow safety procedures resulted in a worker being exposed to a Co-60 source that was used to sterilize medical equipment. The accident resulted in the worker's death 13 days later. At the time of the accident, the consequences of exposure to high levels of radiation were well known. This was not

the case when X-rays were discovered and their use as a diagnostic tool in medicine was beginning to be used. As a result, physicians were exposed to an occupational hazard that they were not aware of. A number of doctors using X-rays daily developed squamous cell carcinomas on their hands and arms. Once a connection was made between X-ray exposure and cancer, steps were taken to protect health care workers and the problem was essentially eliminated.

Patients receive exposure to ionizing radiation when undergoing certain medical diagnostic procedures. The largest increase in exposure to ionizing radiation in the last few decades has come from medical X-rays. According to the National Council on Radiation Protection and Measurement, exposure to radiation for medical purposes has increased from 18% of the total annual exposure in the early 1980s to 48% in 2006 (Table 25.8). The majority of the increase is attributable to X-rays received from diagnostic computer tomography (CT) scans. CT scans represent an important advance in diagnostic procedures, but it requires much larger doses of radiation than conventional X-rays taken with film (Table 25.9). Unlike occupational exposure, medical exposure is not well regulated. A patient might see several different physicians during the year, and each might prescribe a CT scan, resulting in a significant increase in the annual exposure. The increase in exposure to medical X-rays has raised concerns about an increase in the risk of cancer occurring as a latent effect (Brenner, 2007).

Patients are also exposed to radiation for therapeutic purposes. It may seem somewhat ironic to be treating cancer with doses of radiation that can increase one's risk of developing the disease, but it is a matter of risk versus benefit. Fast-growing cells such as tumor cells are more susceptible to being killed by radiation than are normal quiescent cells. Nonetheless, non-cancerous cells are damaged by radiation and therapeutic treatments must be designed to effectively destroy tumor cells while having a minimal impact on normal cells.

### Dose–response curves for accessing risk

There are basically two models that correlate dose versus risk. One presumes that there is a linear relationship between dose and risk with no threshold that must be crossed before a risk exists (curve a in Figure 25.4). This is known as the linear no threshold (LNT) model. The other model posits that below a certain threshold there is no increase in risk with dose but, once the threshold is reached, the risk increases in proportion to the dose (curve b in Figure 25.4). A variation on the latter model (Luckey, 2003) proposed that below the threshold there may be doses of radiation that are beneficial in that these may stimulate or prime the processes within the

**TABLE 25.8** Changes in the annual dose of radiation received by individuals from 1980 to 2006 according to the UNSCEAR 2008 Report to the General Assembly with Scientific Annexes, p. 33

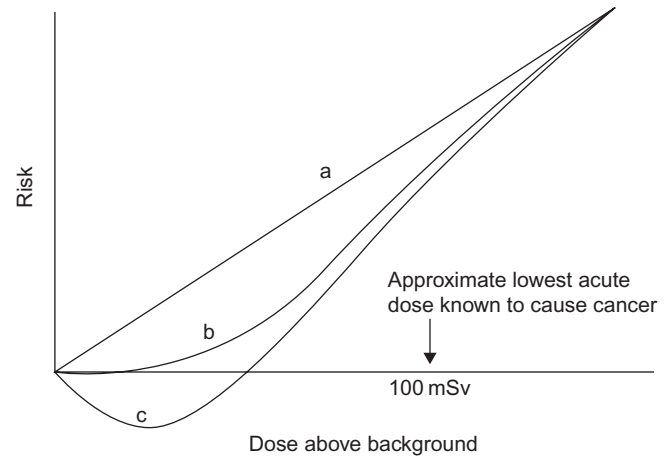
Source	Annual dose in mSv	
	1980	2006
Natural sources	2.4	3.1
All medical sources	0.53	3.0
All other sources	0.05	0.14

**TABLE 25.9** Typical doses of radiation received from medical diagnostic X-rays

Source of X-ray	Dose
<b>Conventional X-rays</b>	
Dental	0.005 mSv
Chest	0.01 mSv
Lateral chest	0.15 mSv
<b>CT scans</b>	
Head	2 mSv
Chest	10 mSv
Abdomen/pelvis	10 mSv
Extremity (hand/foot)	0.1 mSv
Spine	10 mSv
Whole body	10 mSv
Cardiac	20 mSv

cell that are responsible for repairing damage caused by radiation or chemical insults (curve c in Figure 25.4). This phenomenon of priming the repair mechanisms is also known as an “adaptive response” and the beneficial effects are known as radiation hormesis.

The conservative approach is to adopt the LNT model when developing policy to protect individuals from the effects of radiation. However, there are data suggesting that the second model gives a more accurate picture of the situation. Consider the plight of radium dial painters. In the early 1900s, women were employed to paint the hands and dials of clocks and watches with radium paint. The paint contained a mixture of radium radioisotopes and radioluminescent zinc sulfide crystals. As radium decays, it transitions from one radioactive element to another by a series of alpha and beta particle emissions, converting it to a stable isotope of lead. These ionizing particles excite electrons in the zinc sulfide and the electrons emit a photon of visible light when they lose this excess energy. As discussed earlier, most alpha and beta particles emitted by radioactive materials are not very penetrating and can be easily stopped by the glass covering of a watch or in the case of alpha particles by clothing or a few layers of skin cells. However, when ingested or inhaled the source of alpha and beta particles cause damage to the cells they come into contact with. Radium dial painters would tip their paint brushes with their lips to shape them for doing the delicate work at hand and, in so

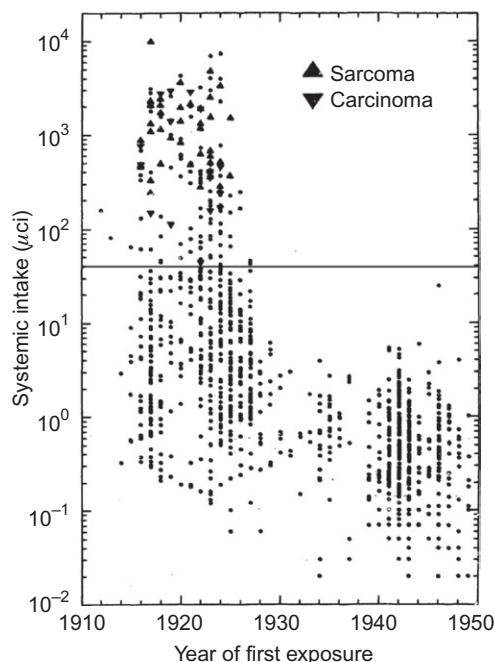


**FIGURE 25.4** Hypothetical dose-response curves for (a) linear no threshold (LNT) response model, (b) threshold model where risk is not increased at low doses of radiation and (c) a model in which low doses of radiation reduce the risk of diseases (radiation hormesis) until a threshold is crossed where increasing dose correlates with increasing risk of developing cancer.

doing, ingested radium. Radium is primarily deposited in bone and many of the women developed bone carcinomas and anemia from radium’s effect on the bone marrow. One hundred and twenty women are known to have died as a result of ingesting radium paint. Once cause and effect was established, a warning was issued to dial painters not to tip their brushes with their lips. Although radium dial painting continued, no dial painter who began work after 1925 when the warning was issued, has been diagnosed with cancer. Nonetheless, dial painters acquired a radioactive burden as can be seen from Figure 25.5 (Rowland and Lucas, 1983) but at a reduced level compared with those women who started painting before 1925. This suggests that there is a dose threshold that must be crossed before there is an increase in the risk of developing cancer. Similar results have been observed in experimental protocols.

Studies have been conducted to evaluate the effects of acute exposure to radiation on the health of individuals living in Hiroshima and Nagasaki, at the time the nuclear bombs were dropped. The combined population of these two cities in August of 1945 was estimated to be 429,000, and 103,000 were killed outright or died within the first 4 months following the blasts. In 1950, there were 283,000 individuals who claimed to have been exposed to radiation from the bombs. A group of these individuals was selected to take place in a Life Span Study Cohort that will continue until 2015. Results obtained thus far suggest that acute effective doses of less than 100 mSv do not increase the risk of developing cancer.

There is accumulating evidence that low levels of radiation can have hormetic effects (Johansson, 2003; Jolly



**FIGURE 25.5** The dose measured as systemic intake in  $\mu\text{Ci}$  (see Tables 25.2 and 25.4 for conversion of  $\mu\text{Ci}$  to Bq) is plotted against the years in which the dial painters began work. The original figure is reproduced here with a horizontal line at  $40 \mu\text{Ci}$  added to indicate the dose below which no dial painters had developed cancer by the time of the report in 1983. Systemic intake is defined as  $\mu\text{Ci}$  of R-226 +  $2.5 \times \mu\text{Ci}$  of R-228. For additional information see Rowland and Lucas (1983). The original report is available at <http://www.osti.gov/bridge/servlets/purl/6374911-rHt6M2/>

and Meyer, 2009). When thymidine is added to the culture medium, lymphocytes will incorporate it into their DNA. If tritium (H-3) is substituted for hydrogen atoms in thymidine, the DNA will be radioactive exposing the cells to low levels of beta radiation. The cells were then exposed to 1.5Gy of X-rays and the extent of chromosomal aberrations determined. Cells that incorporated tritiated thymidine had fewer chromosomal aberrations than did control cells grown in thymidine that was not radioactive, suggesting that a low level of chronic exposure to radiation evokes an adaptive response that protects cells from the effects of subsequent high doses of radiation (Olivieri *et al.*, 1984). Blocking protein synthesis blocked the protective effects of low doses of radiation providing evidence that the mechanism of protection involved inducing protein synthesis, and the evidence suggests that this includes the enzymes that are important for DNA repair. Experimental paradigms analogous to those used to study adaptive response in cultured cells have also been used to investigate the phenomenon in whole organisms like fruit flies (Moskalev *et al.*, 2009) and mice (Mitchel *et al.*, 1999) with similar results. *In vivo* studies indicate that irradiation of mice at a rate 25–50

times the normal background significantly increase lifespan (Caratero *et al.*, 1998), immune competency (Ina and Sakai, 2005) and latency in the onset of spontaneously occurring and induced tumors (Mitchel *et al.*, 2003; Cheda *et al.*, 2004). If the dose of radiation is low enough, no beneficial effects can be demonstrated suggesting that there is a threshold for hormesis (Elmore *et al.*, 2008; Mitchel *et al.*, 2008). In addition to dose, the dose rate and the length of exposure are likely to be important factors in hormesis. Another point to consider is whether different types of cancer have different thresholds for both hormesis and risk.

### Current recommendations regarding exposure limits

Despite the forgoing discussion, studies indicating that low doses of radiation are not detrimental and likely beneficial have not been unequivocally established. Consequently, agencies charged with issuing recommendations and policies regarding radiation exposure such as the International Commission on Radiological Protections (ICRP) and the United Nations Subcommittee on the Effects of Atomic Radiation (UNSCEAR) assume that the LNT model applies. Based on this model, the recommendation is that exposure to ionizing radiation beyond background should be kept “as low as reasonably achievable” (ALARA). The current recommendations on exposure limits are different for those who experience occupational exposure and the general public. The recommendation of the ICRP is that the dose to radiation workers for a 5-year period should not exceed 100 mSv, and no more than 50 mSv should be received in a single year. For radiation workers who become pregnant, the dose to the fetus should be kept below 1 mSv between the time pregnancy is confirmed and the fetus reaches term. The annual dose to the general public should not exceed 1 mSv above the dose from background radiation. In light of accumulating data suggesting that low levels of radiation may not be harmful, and may even be beneficial, the currently recommended limits may need to be revised.

## CONCLUSIONS

Ionizing radiation is a double-edged sword. Released by nuclear weapons it can result in an agonizing death within days, weeks or years of exposure. But ionizing radiation has been a fact of life ever since it evolved on earth more than 3 billion years ago. Although an

appreciation of the potentially devastating effects of radiation arose in the early 20th century with the plight of the radium dial workers, widespread fear of radiation was not established until nuclear weapons were used to destroy Hiroshima and Nagasaki in 1945. Today, the choices we make with regard to the use of radiation require careful consideration. For example, nuclear power carries with it risk that has been made obvious by the accidents at Windscale, Three Mile Island, Chernobyl and Fukushima Dai-ichi, but the human deaths and environmental destruction that have resulted from the use of nuclear power are far less than that associated with the coal industry. In a time when carbon dioxide produced by the burning of fossil fuels is generally accepted as the primary cause of global warming with its potentially disastrous environmental consequences, the ability of nuclear reactors to generate power without producing carbon dioxide makes it an attractive alternative. Today, decisions about radiation extend to the individual choices we make as well. More than ever before, medical diagnostic procedures rely on radiation-based techniques. There are currently no regulations regarding tracking or limiting the total annual exposure received by patients. To an extent, the obligation falls on the individual to weigh risk and benefit. This requires keeping track of exposure and knowledge of safe dose limits. The uncertainty discussed earlier with regard to those limits makes the decision more difficult.

## ACKNOWLEDGMENTS

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# Carcinogenesis: mechanisms and models<sup>1</sup>

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## INTRODUCTION

Animals, like humans, have always been exposed to thousands of chemical substances in their daily lives. This exposure may come from the food they eat, the water they drink and the air they breathe. A high level of exposure to many of these chemicals may cause cancer in humans and animals.

Cancer has become an increasingly prominent disease in recent times but the incidence of cancer had been documented through writings and drawings thousands of years ago. The earliest known descriptions of cancer were found from the writings in Egyptian papyri, discovered and deciphered in the late 19th century. Two of these papyri, which were written around 1600 BC and called "Edwin Smith" and "George Ebers" papyri, contain descriptions of various cancers and Egyptian medical practice of treating cancer. Hippocrates is credited with naming cancer as *karkinoma* (carcinoma) because a tumor looked like a "crab" (*karkinoma* is Greek for crab). Hippocrates believed that the body contained four types of "humors" (body fluids): blood, phlegm, yellow bile and black bile. An excess of black bile collecting in various body sites was thought to cause cancer. The black bile theory of cancer was supported by the influential Greek physician Galen and it dominated scientific thought for over 1300 years. The black bile theory of cancer was eventually replaced by the lymph theory of cancer in the 17th century. The lymph theory was developed from the discovery

of the lymphatic system by the Italian surgeon Gaspare Aselli, and abnormalities of lymph were viewed as the primary cause of cancer. Eventually, the lymph theory gained rapid support. The Scottish surgeon John Hunter (1723–1792) lent support to the lymph theory by hypothesizing that tumors grew from lymph that is constantly thrown out by the blood. It took another 100 years or so before the cellular basis of cancer could be contemplated. The cellular origin of cancer was propounded by Rudolph Virchow in the late 19th century when he recognized that cells, even cancerous cells, were derived from other cells.

In keeping with the theories on the possible physiological causes of cancer, an increasing number of reports from Europe and England documented the association between occupation/chemical exposure and the development of cancer. For example, Paracelsus was probably the first to identify a chemical substance, arsenic disulfide, as the causative agent of lung cancer in the miners of Schneeberg and Joachimsthal, Germany. In 1713, the Italian physician Bernadrino Ramazzini reported the absence of cervical cancer and higher incidence of breast cancer in nuns and thought this was related to their celibacy. The English physician Percival Pott observed that young men in their twenties who had been chimney sweeps had a high rate of cancer of the scrotum. He suggested that the causative agent might be chimney soot (tar) and recommended frequent washing and changing of clothes in order to reduce exposure to the soot. John Hill (1761) in the U.K. implicated the use of tobacco as snuff in the development of nasal polyps. In 1795, Samuel Thomas von Soemmering in Germany reported the occurrence of lip cancer among pipe smokers. In 1895, Ludwig Wilhelm Carl Rehn reported the occurrence of bladder cancer among workers in the

<sup>1</sup>The opinions expressed in this article are the authors' personal opinion and they do not necessarily reflect those of FDA, DHHS or the federal government.

German dye industry. Various epidemiological studies have since identified major environmental causes of cancer, which include both naturally occurring chemicals as well as man-made chemicals.

Experimental cancer research began in the early 20th century and has been continuing since. Recent advances in molecular genetics have provided researchers with additional tools to study the mechanisms and the molecular biology of cancer. The knowledge gained from such studies form the foundation of our understanding of the process of carcinogenesis. Today, chemical carcinogenesis is regarded as a multistep process with a long latency period. For a relatively detailed historical perspective of cancer, readers are referred to [Marquardt \(1999\)](#), [Pitot \(2002\)](#) and the American Cancer Society website.

## TERMINOLOGY

*Cancer* is a term that is commonly used to indicate a group of diseases characterized by uncontrolled cell proliferation and usually the spread of these abnormal cells. In common parlance, the word cancer is near-synonymous with other expressions such as *malignant tumor*, *malignant neoplasm*, *malignancy* and *neoplasia*. Tumors or neoplasms (new growth of tissue) can thus be benign or malignant (cancerous). In this chapter, these expressions will be used interchangeably. Agents causing cancer are called *carcinogens* and the process of cancer development is called *carcinogenesis*. Most neoplasms arise from the clonal expansion of a single cell that has undergone neoplastic transformation. Hence, cancers are monoclonal. According to [Pitot \(2002\)](#), *neoplasia* or the constituent lesion *neoplasm*, can be defined as a heritably altered, relatively autonomous growth of tissue. The “heritably altered” aspect reflects the abnormal genetic expression, which is either inherent in the neoplastic cells or is induced in response to environmental stimuli. Another term relevant to cancer is hyperplasia. *Hyperplasia* means an increase in cell number. Hyperplasia can be found in neoplastic as well as non-neoplastic conditions. Examples of non-neoplastic hyperplasia include callus formation, hepatomegaly (liver enlargement), etc. Hepatomegaly in response to various xenobiotic treatments usually involves both hyperplasia and hypertrophy (enlargement of cells).

In the parlance of cancer biology, the suffix “oma” means tumor, benign or malignant. For example, *fibroma* and *lipoma* are benign neoplasms, while *melanoma*, *hepatoma* and *seminoma* are malignant neoplasms. Sometimes even non-neoplastic lesions end with the “oma” suffix, such as *hematoma* and *granuloma*. A hematoma is a localized collection of blood outside the blood vessels, usually in liquid form within the tissue. This happens when blood escapes

into the surrounding tissue because of damage to the blood vessels. Likewise, a granuloma is a small patch of inflamed tissue that results from infection or inflammation. Cancer of the embryonic tissue is denoted with the suffix “blastoma,” such as *neuroblastoma* and *retinoblastoma*. Similarly, cancer of connective tissue, such as bone, cartilage, fat, muscle and blood vessels, is denoted with the suffix “sarcoma,” such as *fibrosarcoma*, *liposarcoma* and *rhabdomyosarcoma*.

## CANCER EPIDEMIOLOGY

Cancer incidence data are available in many countries from registries that monitor the occurrence of different types of cancers in various populations. Epidemiological data show that certain cancers tend to occur more frequently among people in certain geographic locations. For example, breast and prostate cancers have lower incidence rates in Asian countries but higher incidence rates in Europe and North America ([Haas and Sakr, 1997](#); [McPherson et al., 2000](#)). In contrast, gastric cancer has lower incidence rate in economically developed western countries including the U.S., but higher incidence in Japan and Korea ([Alberts et al., 2003](#)). These differences in cancer incidence reflect the role of both genetic and environmental factors. Strong evidence for the importance of environmental factors comes from studies on migrant populations. When there is a significant difference in the incidence of a specific cancer in the native country and the new host country, the migrant population acquires the cancer incidence rate of the host country. For example, Japanese populations in western countries typically acquire the higher breast and colon cancer rates of the host country within a generation or two. Incidence of cancer may also be influenced by gender. For example, gastric cancer shows about a two-fold higher incidence in males than females in every region of the world studied ([Alberts et al., 2003](#)). While knowledge of the genetic basis of cancer helps us understand the molecular mechanism of carcinogenesis, knowledge of cancer-causing environmental factors is crucial to making important health and environmental policy decisions that may have far-reaching impact on human and animal health.

## AGENTS CAUSING CARCINOGENESIS

The three main classes of agents (carcinogens) causing cancers are chemicals, radiation and viruses. In this chapter, chemical carcinogenesis is emphasized, while viral and radiation carcinogenesis is also discussed briefly. Recent research also shows an important role of epigenetics in carcinogenesis.

**TABLE 26.1** Some known/suspected chemical carcinogens and their target organs

Chemicals/suspected carcinogens	Organs affected
Aflatoxin	Liver
4-Aminobiphenyl	Bladder
Arsenicals	Lung, skin
Diesel exhaust	Lung
Benzene	Leukemia
Cigarette smoking	Lung
Pipe smoking	Lip
Soot	Scrotum
Dyes (aromatic amines, such as 2-naphthylamine)	Urinary bladder
Nickel compounds	Lung
Vinyl chloride	Liver
Radium (radioactive watch colors)	Bone
Formaldehyde	Nose
Snuff	Nose
Diethylstilbestrol	Genital tract

Source: Marquardt (1999)

## Chemical carcinogenesis

### Chemical carcinogens

Chemical carcinogens originate from both industrial and natural processes. In 1915, Yamagawa and Ichikawa pioneered the field of experimental cancer research when they produced tumors by repeated application of coal tars on the skin of rabbits. Many of the chemical carcinogens identified in early research were byproducts of industrial processes. Subsequent studies have revealed the carcinogenic potential of many other industrial, naturally occurring chemicals, as well as man-made chemicals. Chemical carcinogens may be synthetic (man-made) or of natural origin; they are extremely diverse in structure and belong to very different chemical classes, such as inorganic, organic, fibers, plastic, hormones, etc.

Based on their biological activities, chemical carcinogens can be classified as *genotoxic carcinogens* (DNA reactive) and *non-genotoxic carcinogens* (non-DNA reactive, epigenetic). Genotoxic carcinogens can be further classified as *direct carcinogens* (active without metabolic activation) and *indirect carcinogens* or *procarcinogens* (active after metabolic activation). Table 26.1 lists some of these chemical carcinogens and the organs affected by them.

### Biological process of chemical carcinogenesis

Experimental cancer research with carcinogenic chemicals led to the realization that chemical carcinogenesis is a multistep process. The pathogenesis of cancer involves many individual events, such as metabolic activation (biotransformation) of a procarcinogenic chemical to a DNA-reactive compound (in the case of indirect

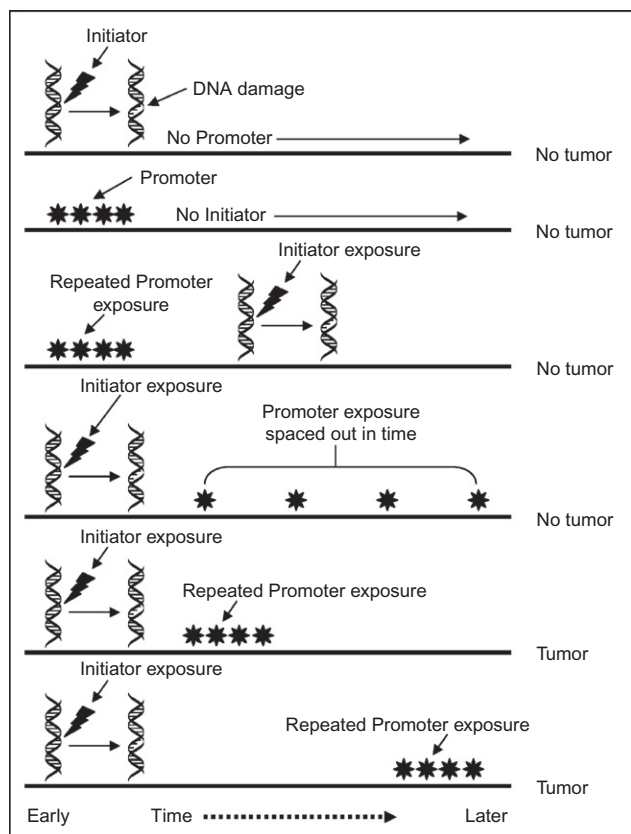
genotoxic carcinogens), covalent binding to DNA, induction of heritable mutations and finally expression of the mutant phenotype at the molecular level (e.g., altered gene expression). All these changes result in altered cellular function, cellular transformation and neoplastic growth, and spreading of the transformed cells to other parts of the body. These events in the pathogenesis of cancer have been distilled into three distinct steps to describe the multistep nature of carcinogenesis; the steps are *initiation*, *promotion* and *progression* (Foulds, 1954). *Tumor initiation* involves introduction of heritable genetic change in a normal cell; *tumor promotion* involves clonal expansion of the initiated cell resulting in the formation of a benign tumor; and *tumor progression* involves the conversion of a benign tumor into a malignant one. Malignancy in cancers is characterized by anaplasia (reversion of cellular differentiation), invasiveness and metastasis.

In order for the tumors to develop, *the target tissue must be exposed to an initiator first and then repeatedly exposed to promoters*. If the time gap between the exposure to initiator and promoter varies from a week to a year, tumors will still develop. However, if the exposure to promoter is first and is followed by exposure to initiator, tumors will not develop. After the promotion event, the cancer cell acquires further heritable changes that lead to malignancy. Figure 26.1 shows the importance of the sequence of exposure to tumor initiator and tumor promoter in the development of cancer.

### Initiation

In the initiation phase, the chemical or its reactive metabolite causes a permanent change in the DNA of the target cell(s), such as a mutation, a distortion of the DNA structure with further consequences, elimination of a component of DNA (bases or sugars) or errors in DNA repair (Pitot, 2002). Thus, initiation is a genetic process. The chemical causing initiation is called an *initiator*. Once a target cell is initiated, initiation is irreversible. An initiated cell by itself is not a cancer cell because it has not acquired the property of uncontrolled growth. Additionally, all initiated cells do not produce tumors because many undergo programmed cell death or *apoptosis*. In order for an initiated cell to transform into a cancer cell and eventually produce a detectable tumor, promotion is necessary.

Some examples of tumor initiators are benz[a]pyrene; 7,12-dimethylbenz[a]anthracene; 3-methylcholanthrene; 2-acetylaminofluorene; dimethylnitrosamine; diethylnitrosamine, etc. The mechanism of tumor initiation by many initiators involves the formation of depurinating adducts, that is, the initiator forms an adduct with the purine bases, and removal of these purine adducts ultimately leads to a transition or transversion mutation in



**FIGURE 26.1** The target tissue must be exposed to an initiator first, followed by repeated exposure to promoters in order for the tumors to develop. If the time gap between the exposure to initiator and promoter varies from a week to a year, tumors will still develop. However, if the exposure to promoter is first and is followed by exposure to initiator, tumors will not develop. Also, if the exposure to promoter is not repeated and spaced over time, tumors are not likely to develop even if the exposure to promoter occurs after exposure to initiator.

the DNA. For example, 7,12-dimethylbenz[a]anthracene (DMBA) involves the formation of adducts in which DMBA is bound through its 12-methyl group to the N-7 position of adenine (major adduct) or guanine (minor adduct). Removal of the N-7 purine adducts leads to depurination and mutation, such as A→T transversion in *ras* oncogene. Some DNA lesions can be repaired. For example, exposure to the tumor initiator *N*-methyl-*N*-nitrosourea (MNU) results in the modification of guanine into *O*<sup>6</sup>-methylguanine in the DNA of the target tissue. Failure to repair the *O*<sup>6</sup>-methylguanine lesion leads to G→A transition. The "suicide enzyme" *O*<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) repairs this lesion. Transgenic mice overexpressing MGMT are less sensitive to tumor development in the tissue overexpressing the enzyme because of the increased repair of *O*<sup>6</sup>-methylguanine (Becker *et al.*, 1996, 2003; Liu *et al.*, 1999; Qin *et al.*, 2000). Experiments with

MGMT transgenic mice have also demonstrated that the *O*<sup>6</sup>-methylguanine lesion is possibly also involved in tumor progression (Qin *et al.*, 2000; Becker *et al.*, 2003).

Although a methyl group is the preferred substrate, MGMT can also repair DNA lesions by removing larger and complex alkyl groups, such as ethyl, propyl, butyl, benzyl and 2-chloroethyl groups, which makes MGMT an alkyltransferase rather than a methyltransferase. Repair of the *O*<sup>6</sup>-methylguanine by MGMT involves a cysteine residue in its active site (Cys145 in human MGMT). The methyl group becomes covalently attached to this cysteine residue, and this modification results in the inactivation of MGMT, which is then ubiquitinated and degraded by the proteasome. Hence MGMT's name – "suicide enzyme." Removal of other alkyl groups occurs through the same mechanism. Because MGMT is a suicide enzyme, continued DNA repair by MGMT requires *de novo* MGMT synthesis.

### Promotion

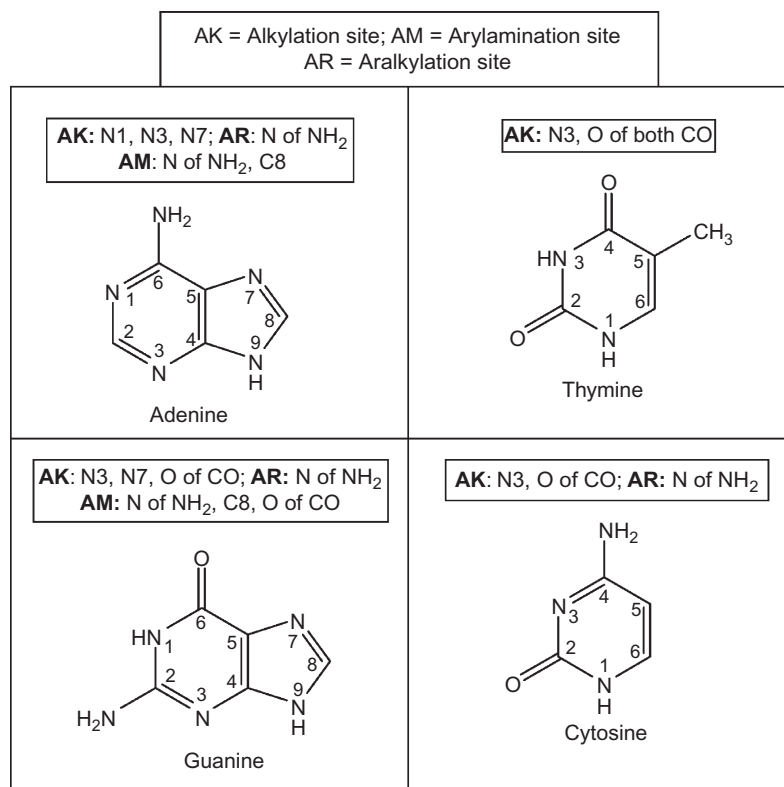
Tumor promoters alter the expression of genetic information of the cell, as well as, in many cases, inhibit programmed cell death (Pitot, 2002). Tumor promoters usually influence the proliferation of initiated cells, which results in the proliferation of preneoplastic cells and formation of benign focal lesions, such as enzyme-altered foci in the liver, nodules in the mammary gland, polyps in the colon and papillomas in the skin. Because tumor promoters alter the expression of genetic information without changing the DNA sequence, tumor promotion is an epigenetic process. In addition to causing cell proliferation, tumor promoters appear to block apoptosis, thus leading to accumulation of the initiated cells as dysfunctional, non-differentiated cells within a tissue. Some of the lesions that develop due to promotion regress, but others acquire additional mutations and progress to malignant neoplasm. Tumor promotion is a reversible process up to a certain stage if the promoter is withdrawn.

Tumor promoters tend to be organ specific. For example, TPA (12-*O*-tetradecanoylphorbol-13-acetate, a phorbol ester isolated from croton oil) is skin specific. Chlordane, DDT (dichlorodiphenyltrichloroethane), TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), phenobarbital,  $\beta$ -naphthoflavone, peroxisome proliferators and polybrominated biphenyls are hepatic tumor promoters; mirex (an organochlorine) is a promoter in both skin and liver; saccharin is a bladder tumor promoter.

### Progression

Tumor progression involves autonomous growth and metastasis of the malignant neoplasm. At the cellular level, chromosomal breakage and deletion, duplication or translocation of chromosomal fragments are





**FIGURE 26.2** DNA bases showing the sites where alkylation, arylamination and aralkylation reactions can occur resulting in the formation of DNA adducts (according to Dipple, 1995). N3 alkylation of guanine, although reported in the literature and shown in the figure, is less common than N7 alkylation.

hallmarks of tumor progression phase. Additional mutations in the oncogenes and tumor suppressor genes also accumulate during this phase. This is why tumor initiators can also cause tumor progression (Qin *et al.*, 2000; Becker *et al.*, 2003). Karyotypic instability and accumulation of additional mutations may reflect an ongoing selection of cells suitable for neoplastic growth and metastasis (Okey *et al.*, 1998).

Some putative tumor progression agents (with no initiator activity) that cause transition from promotion to progression include benzene, benzoyl peroxide and 2,5,2'5'-tetrachlorobiphenyl (Okey *et al.*, 1998).

#### Mode of action of chemical carcinogens

Both direct and indirect carcinogens (procarcinogens) interact with DNA, forming DNA adducts. Indirect carcinogens (procarcinogens) acquire carcinogenic properties after metabolic activation, forming the *ultimate carcinogen*, which is the reactive metabolite that covalently modifies DNA. The biological activity of the carcinogens depends on a balance between their activation and detoxification in the target tissue.

The majority of these compounds interact with DNA through three different types of chemical reactions (Dipple, 1995; Okey *et al.*, 1998). The reactions involve the transfer of (1) an alkyl group, (2) an arylamine group or (3) an aralkyl group to DNA. An arylamine contains an amine-substituted aromatic ring, while an aralkyl

(arylalkyl) contains an alkyl-substituted aromatic ring. An example of a reactive arylamine group is arylnitrenium ion while an example of a reactive aralkyl group is benzyl radical.

The DNA-reactive groups are generated through specific reaction chemistries, such as oxidation at carbon-carbon double bonds yielding alkylating or aralkylating agents; oxidation or reduction at nitrogen producing arylaminating agents; conjugations of hydroxy compounds producing aralkylating or arylaminating agents; and conjugation between glutathione with dihaloalkanes producing alkylating agents. There are, however, some carcinogens that do not fit these categories, such as acylating agents,  $\alpha,\beta$ -unsaturated aldehydes, chloroethylene oxide, etc. (Dipple, 1995).

#### Alkylation and alkylating agents

Alkylating agents add an electrophilic alkyl group ( $R-CH_2^+$ ), a carbocation, to electron-rich (nucleophilic) sites in DNA. Carcinogens that transfer alkyl residues to DNA include nitrosamines, aliphatic epoxides, aflatoxins, lactones, nitrosoureas, mustards, haloalkanes, aryl triazenes and sultones (Dipple, 1995).

The sites of substitution in DNA bases by alkylating agents are many and are indicated in Figure 26.2. Alkylating agents are produced by enzymatic reactions (e.g., P450 mediated). An example is the cytochrome P450 2E1 (CYP2E1)-mediated metabolism of

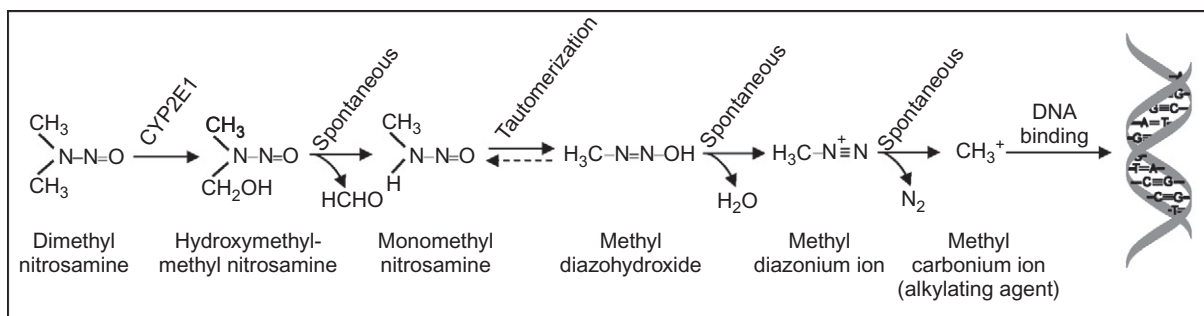


FIGURE 26.3 Metabolic activation pathway for dimethylnitrosamine.

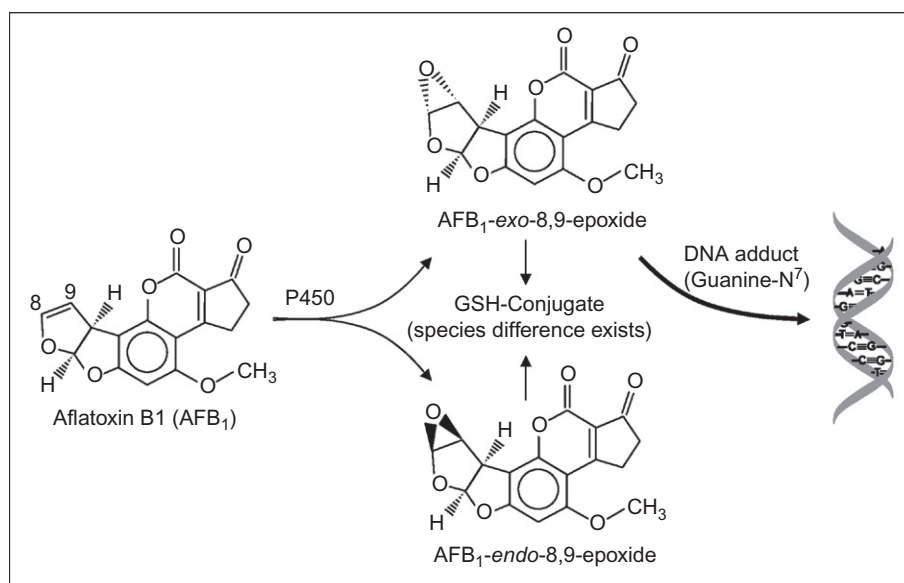


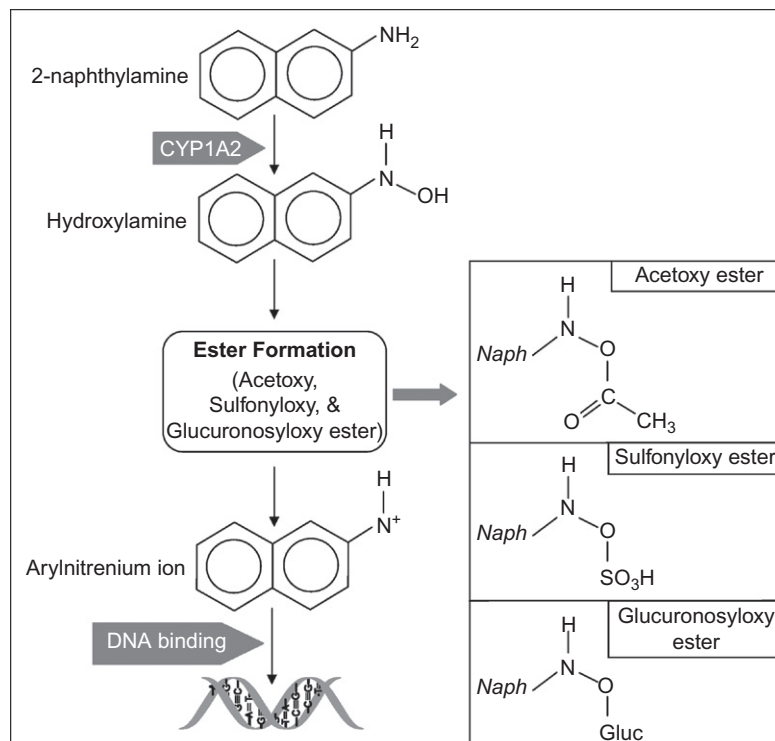
FIGURE 26.4 Metabolic activation pathway for aflatoxin B1.

dimethylnitrosamine (DMN) (Figure 26.3). CYP2E1-mediated hydroxylation of a methyl group in DMN results in the formation of an unstable intermediate hydroxymethyl-methyl nitrosamine, which undergoes spontaneous demethylation forming monomethylnitrosamine (MMN) and releasing one molecule of formaldehyde. The MMN undergoes tautomerization to form methyl diazohydroxide, which undergoes spontaneous decomposition to first form the methyl diazonium ion and then methyl carbonium ion (CH<sub>3</sub><sup>+</sup>). The methyl carbonium ion is the ultimate highly reactive alkylating agent that methylates nucleophilic sites on DNA and proteins. Larger *N*-nitroso compounds, such as tobacco smoke-derived NNK ((4-methylnitrosamino)-1-(3-pyridyl)-1-butanone), are activated by cytochrome P450 1A2 (CYP1A2). Aflatoxin B1 (AFB<sub>1</sub>) is also bioactivated by P450. In humans, cytochromes P450 1A2 (CYP1A2) and P450 3A4 (CYP3A4) appear to be the most important in the metabolic activation of AFB<sub>1</sub> (Van Vleet *et al.*, 2002). Hydroxylation of the unsaturated C<sub>8</sub>-C<sub>9</sub> bond in AFB<sub>1</sub> results in the formation of a reactive epoxide intermediate, the 8,9 epoxide (Figure 26.4).

The AFB<sub>1</sub>-exo-8,9-epoxide is the most relevant epoxide; it forms DNA adducts almost exclusively at the N-7 position of guanine. This results in a GC→TA transversion mutation at the third position of codon 249 of the *p53* gene, which is thought to be a major mechanism of aflatoxin-induced carcinogenesis (Smela *et al.*, 2001).

#### Arylamination and arylaminating agents

Aromatic amines (arylamines), amides, aminoazo dyes, heterocyclic amines, etc. all undergo metabolic activation forming highly reactive, electrophilic aryl nitrenium ions (Ar-NH<sup>+</sup>). The major sites of substitution in DNA by arylaminating agents appear to be the C-8 position and the amino group of the purine nucleotides (Dipple, 1995). Potential arylamination sites in DNA bases by arylaminating agents are indicated in Figure 26.2. A prototypical example is the metabolic activation of 2-naphthylamine. *N*-oxidation by CYP1A2 forms the corresponding *N*-hydroxyarylamine (e.g., *N*-hydroxynaphthylamine), which can undergo a number of conjugation reactions forming sulfate,



**FIGURE 26.5** Metabolic activation pathway for 2-naphthylamine.

acetate or glucuronide conjugates. These conjugates can be excreted in the urine. In the acidic pH of urine, the conjugate dissociates and the *N*-hydroxynaphthyl moiety is protonated to form the nitrenium ion (Figure 26.5). Formation of the nitrenium ion, which is the ultimate carcinogen, explains why 2-naphthylamine is carcinogenic in the urinary tract and urinary bladder. *N*-acetyltransferase (NAT), which catalyzes the transfer of acetyl groups from acetyl-CoA to arylamines forming acetyl esters, shows a great deal of polymorphism across species. Among humans, there are slow and fast acetylators. In hamsters, acetylation is fast while in dogs and rats, acetylation is slow. These species differences in *N*-acetyltransferase activity as well as intra-species polymorphism can greatly influence the toxicity outcome of the substances in question.

#### *Aralkylation and aralkylating agents*

Polycyclic aromatic hydrocarbons (PAHs) and related compounds are capable of transferring an aralkyl group to DNA. Carcinogens that transfer an aralkyl group to DNA include the polycyclic aromatic hydrocarbons, alkyl benzenes, pyrrolizidine alkaloids and nitroaromatics that are activated through the formation of dihydrodiol epoxide (Dipple, 1995). A classic example of this class is benzo[a]pyrene, which is converted by cytochrome P450 1A1 (CYP1A1) to benzo[a]pyrene 7,8 epoxide. This epoxide is readily converted by epoxide hydrolase to the 7,8 dihydrodiol of benzo[a]pyrene. Benzo[a]pyrene 7,8 dihydrodiol, in

turn, is converted by CYP1A1 to the 7,8 dihydrodiol-9,10 epoxide of benzo[a]pyrene. This 9,10 epoxide is part of the bay region, electrophilic and resistant to hydration by epoxide hydrolase; therefore it readily attacks DNA bases, covalently binds DNA and induces mutation, such as mutation in the 12th codon of *H-ras* oncogene (Figure 26.6). Potential aralkylation sites in DNA bases by aralkylating agents are indicated in Figure 26.2.

#### *Cellular defense against DNA damage – DNA repair and removal of DNA adducts*

DNA adducts (the chemical-induced, covalently modified DNA bases) can alter the DNA structure and, in turn, molecular processes, such as replication and transcription. If not repaired or repaired incorrectly, these modifications may ultimately lead to mutations and eventually cancer, particularly if the adduct is located in an oncogene or tumor suppressor gene. There are a number of mechanisms to repair DNA adducts: (1) direct repair, (2) base excision repair (BER), (3) nucleotide excision repair (NER) and (4) mismatch repair (MMR).

In *direct repair*, the bond between the nucleotide and the adduct is broken, restoring the original conformation. For example, *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-MeG) is repaired by MGMT (methylguanine DNA methyltransferase) by direct demethylation of the methylguanine. If methylguanine is not repaired, it will pair with thymine instead of cytosine during replication. In the following replication cycle, this thymine will pair with adenine in the

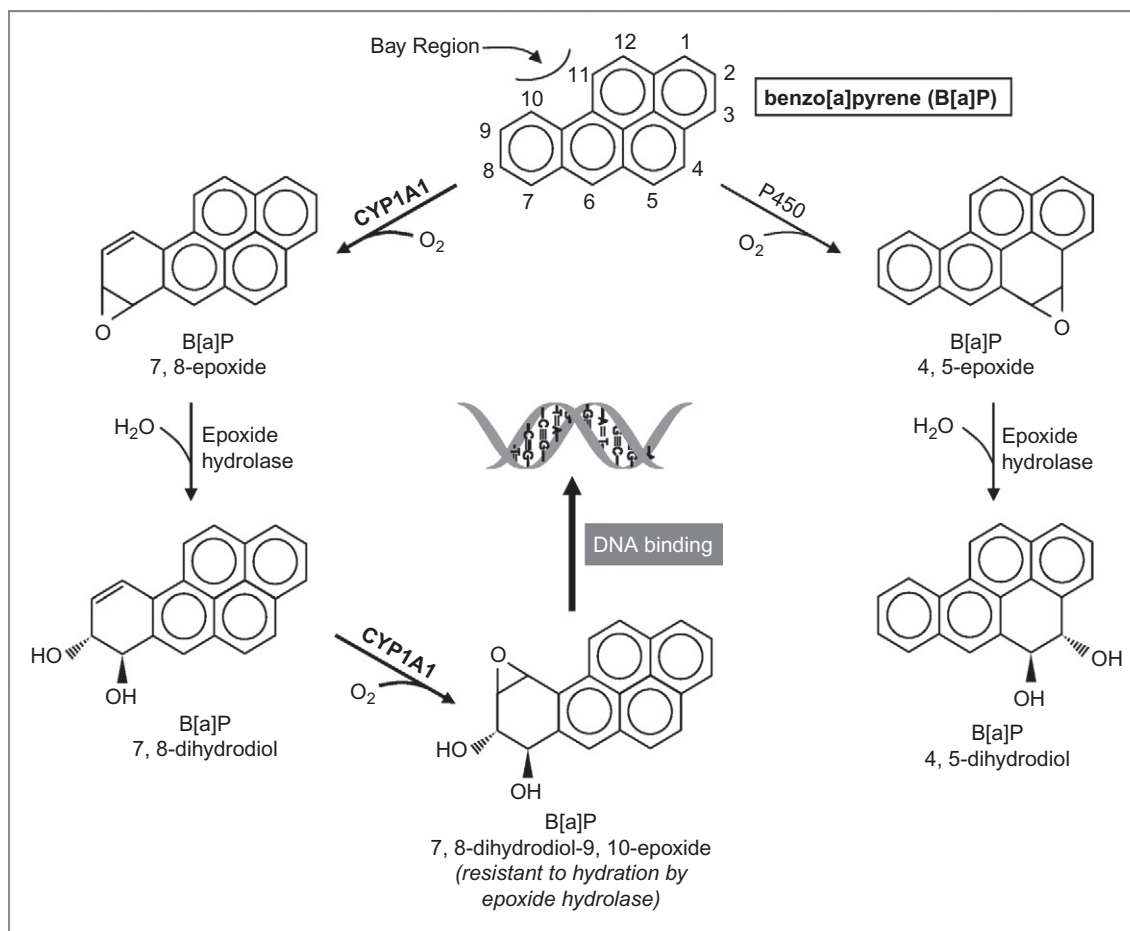


FIGURE 26.6 Metabolic activation pathway for benzo[a]pyrene.

complementary strand. The net result is a G:C→A:T transition mutation in the DNA. Such G:C→A:T transition mutations are known to activate the *ras* oncogenes in nitrosourea-induced mouse lymphomas and rat mammary tumors, as well as in azoxymethane (AOM)-induced aberrant crypt foci (ACF) (Dumenco *et al.*, 1993; Zaidi *et al.*, 1995). In animals that have photolyase, thymine dimers are also repaired by the direct repair mechanism that involves breaking of the bond causing the lesion.

*Base excision repair (BER)* mechanism works mainly on non-bulky base modifications such as those produced by methylation (in positions where it does not interfere with H-bonding, such as 3- or 7-methyladenine or 8-hydroxyguanine). These types of methylation patterns are produced by MNU (*N*-methyl-*N*-nitrosourea). These inappropriate bases are recognized and removed by specific DNA glycosylases, which cleave the *N*-glycosidic bond between the base and the sugar. As a result, the base is removed and the sugar-phosphate backbone of the DNA remains intact. This creates a base gap in the DNA, also called an AP site (apurinic or apyrimidinic

site). The AP site is then recognized by AP endonuclease, which introduces a nick 5' to the AP site. A third enzyme, deoxyribophosphodiesterase (an excision endonuclease), then produces a second nick 3' to the AP site and removes the base-less sugar-phosphate backbone. The resultant one nucleotide gap is filled in by DNA polymerase, and DNA ligase seals the gap.

*Nucleotide excision repair (NER)* is a versatile mechanism that can eliminate a wide range of structurally unrelated lesions. Bulky base modifications such as benzo[a]pyrene-guanine adducts caused by smoking, cisplatin-guanine adducts and psoralen-thymine adducts resulting from chemotherapy, UV-induced multiple thymine dimers, etc. are all repaired by NER. In humans, thymine dimers are repaired by NER because humans do not have photolyase. The mechanism of NER involves generation of two nicks by an excision nuclease, one on each side of the lesion. As a result, a small portion of the affected DNA strand is removed. DNA polymerase then continues the repair synthesis and ligase seals the gap.

*Mismatch repair (MMR)* mechanism repairs bases that violate Watson-Crick base pairing rules. The classic



example is that of *E. coli*. The sequence 5'-GATC-3' in *E. coli* DNA is methylated at adenine, and the sequences 5'-CCAGG-3' and 5'-CCTGG-3' are methylated at cytosine. When DNA replicates, the daughter strand methylation is delayed. As a result, the newly synthesized daughter strand is always undermethylated compared to the parental strand. If there is a base misincorporation, the MMR machinery (MutS-MutH-MutL complex) identifies the misincorporated base by scanning the methylation status of both strands. The mismatched base is excised from the undermethylated daughter strand. The same principle applies to eukaryotic DNA mismatch repair. Three eukaryotic protein heterodimers composed of Msh (MutS homolog) subunits recognize DNA mismatches with different but overlapping specificities: Msh2·Msh3, Msh2·Msh6 and (only in plants) Msh2·Msh7. A second heterodimer, Mlh1 (MutL homolog1)·Pms2, couples mismatch recognition to excision of the error-containing nascent DNA. Deficiencies in Msh2 dramatically increase mutation rates (Hoffman *et al.*, 2004). For example, in HNPCC (hereditary nonpolyposis colorectal cancer) and other human cancers, mutations in MMR genes, such as MSH2, MSH6, MLH1 and PMS2 have been observed (Buermeier *et al.*, 1999). Consistent with this finding, mice transgenic for Msh2 are tolerant to the carcinogenic effects of methylating agents (deWind *et al.*, 1995).

Apart from the above-mentioned mechanisms, two other mechanisms of DNA repair are recombination repair and strand break repair. In *recombination repair*, when DNA polymerase encounters an unrepaired lesion on the parental (template) strand during replication, it stops synthesis at that point and resumes synthesis at the next priming site. This leaves a gap in the daughter strand. This gap is filled by recombination with the other parental strand. This donor parental strand is then repaired by polymerase and ligase. *Strand break repair* may involve single or double-strand breaks. *Single-strand breaks* do not disrupt the integrity of the DNA. The intact single strand is coated by PARP 1 (Poly(ADP-ribose) polymerase-1) protein near the lesion site of the other strand. The single-strand break is then repaired as in excision repair. *Double-stranded breaks* are dangerous because they damage the integrity of the DNA. Double-stranded breaks can be repaired in two ways; either by crossing over or by synthesis-dependent strand-annealing repair. Various proteins (such as Ku) bind to the broken ends of the DNA to protect it and initiate the repair.

## Viral carcinogenesis

### Tumor viruses, oncogenes and tumor suppressor genes

A number of cellular genes are now implicated in carcinogenesis. These genes are of two types; oncogenes

and tumor suppressor genes. Broadly speaking, activation of oncogenes and inactivation of tumor suppressor genes may have similar consequences in terms of carcinogenesis. Oncogenes are found in cancer-causing viruses, which are of two types; DNA viruses with DNA genomes and RNA viruses with RNA genomes. DNA viruses with oncogenic potential are from six distinct viral families: hepatitis B viruses, simian virus 40 (SV40) and polyomavirus, papillomaviruses, adenoviruses, herpesviruses and poxviruses. In contrast, members of only one family of RNA viruses, the retroviruses, are capable of inducing oncogenic potential (Cooper, 1995).

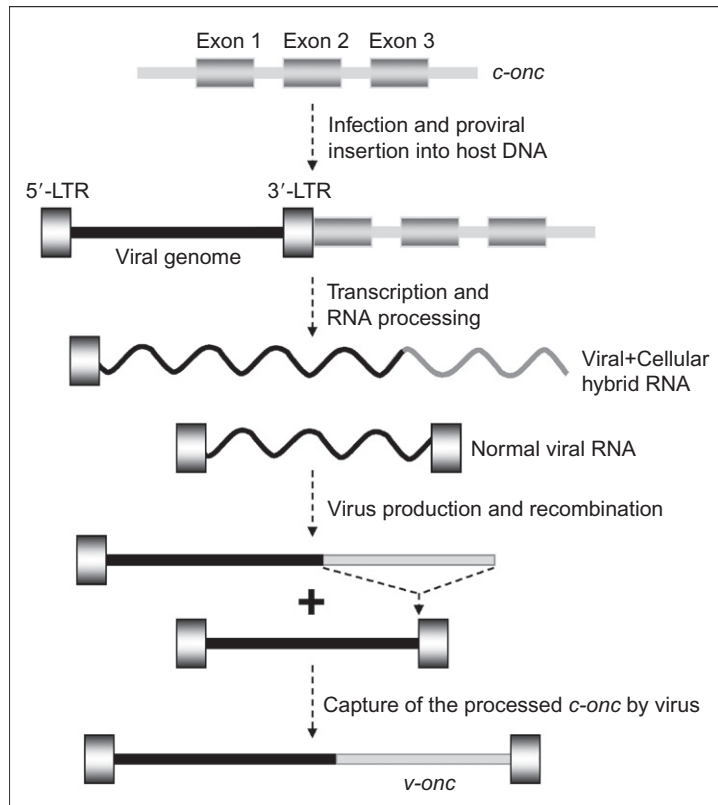
### Retroviral oncogenes and their evolution

The discovery of retroviral oncogenes significantly advanced our understanding about the function of genes responsible for the induction of abnormal cell proliferation. They replicate inside the cell through a DNA intermediate, called provirus, which is integrated into the chromosomal DNA of the infected cell. Transforming viruses (viruses capable of inducing malignant transformation of animal cells) do not need the oncogene for their replication. Viral oncogenes (*v-onc*) are derived from cellular proto-oncogenes (*c-onc*). Cellular proto-oncogenes contain introns while the corresponding viral oncogenes generally lack introns. Thus viral oncogenes originated in the host through recombination between the genome of a non-transforming retrovirus and a cellular oncogene. This was demonstrated in studies on the *v-src* oncogene by Hanafusa and colleagues in the early 1980s.

Figure 26.7 shows how a cellular proto-oncogene (*c-onc*) could be acquired by the viral genome. Transforming viruses preferentially integrate at the fragile sites on the host genome. These fragile sites correspond to the hot spots of recombination. Integration of the provirus in the genome next to the *c-onc* produces a transcription unit under the control of the viral LTR (long terminal repeats). Transcription of this unit produces a viral/cellular hybrid RNA. Intact viral RNAs are also produced from other integrated proviruses. These intact viral RNAs and hybrid RNAs, through reverse transcription and recombination, capture the processed cellular proto-oncogene as part of the viral genome. The captured processed cellular proto-oncogene now becomes a retroviral oncogene (*v-onc*).

### Activation of oncogenes

Retroviral oncogenes (*v-onc*) are altered versions of normal cellular genes called proto-oncogenes. The function of the *v-onc* product is similar or identical to that of the proto-oncogene (*c-onc*) product, but its expression is generally unregulated. Proto-oncogenes may be activated by mutation, chromosomal rearrangement or



**FIGURE 26.7** Mechanism by which a cellular proto-oncogene (*c-onc*) is captured by retrovirus to give rise to a viral oncogene (*v-onc*).

gene amplification. Chromosomal rearrangements, such as translocations and inversions, can activate proto-oncogenes by dysregulation of their transcription (e.g., transcriptional activation) or by gene fusion. For example, the product of the *ras* oncogene differs from that of the normal proto-oncogene by one base which is caused by a point mutation in the 12th codon (GGC→GTC) resulting in one amino acid change (Gly→Val). An example of oncogene activation through gene amplification is *myc*, which codes for a transcription factor that plays a role in cell division. One third of all neuroblastomas show *myc* oncogene amplification that inversely correlates with the prognosis. Such amplification of the *myc* oncogene results in the generation of a very high amount of its product. Generation of a high amount of *myc* oncogene product can also be due to high levels of transcription. This has been reported in Burkitt's lymphoma where translocation of the *myc* proto-oncogene from its normal location (chromosome 8) to chromosome 14 brings it close to the immunoglobulin heavy chain gene promoter. As a result, *c-myc* now finds itself in a region of vigorous transcriptional activity, with a consequent overproduction of its product. Another example of chromosomal translocation and dysregulation of proto-oncogene is found in chronic myelogenous leukemia (CML). In CML, a reciprocal translocation occurs between chromosomes 9 and 22 [t(9;22)]. A portion on the long arm of chromosome 9 (9q) containing the *abl* gene is

translocated next to the *bcr* (*breakpoint cluster region*) gene on the long arm of chromosome 22 (22q). The altered chromosome 22 is called the Philadelphia chromosome (Ph'). The *bcr-abl* produces higher levels of a fusion protein Bcr-Abl, which has constitutive protein tyrosine kinase activity in the Abl portion of the protein. The Bcr portion of this fusion protein can bind to various adaptor molecules. As a result, this fusion protein can associate with many proteins involved in the mitogenic signaling pathway, causing activation of mitogenic signaling and increased cell proliferation. Some other oncogenes associated with cancers of various organs are *hst* (stomach cancer), *met* (osteosarcoma), *bcl-1* (B cell leukemia), etc.

#### *Inactivation of tumor suppressor genes*

Tumor suppressor genes, which also participate in the regulation of normal cell growth, are usually inactivated by point mutations or truncation of their protein sequence coupled with the loss of the normal allele. The first mutation may be inherited or somatic. The second mutation will often be a gross event leading to loss of heterozygosity and tumor suppressor function. This mechanism provides support to the two-hit hypothesis in which he applied statistical analysis to compare patients with hereditary and nonhereditary forms of retinoblastoma. Knudson hypothesized that one germ

line copy of the damaged gene that is inherited in the hereditary form of retinoblastoma was not sufficient to trigger the development of this cancer. A second hit to or loss of the good copy in the gene pair was necessary to produce retinoblastoma. Frequent loss of heterozygosity in the tumor cells provides support to Knudson's hypothesis. Analysis of linkage markers in retinoblastoma cells and normal cells from the same individual shows that the markers that are heterozygous in normal cells are hemizygous in tumor cells, indicating the loss of the normal allele in the tumor cells (second hit).

#### *Functions of oncogene and tumor suppressor gene products*

Oncogene products are called oncoproteins. Proto-oncogenes encode proteins that are mostly involved in the regulation of cell growth, division and differentiation. Consequently, proto-oncogenes encode products that can act as growth factors, growth factor receptor-associated tyrosine kinases, membrane-associated non-receptor tyrosine kinases, G-protein-coupled receptors, membrane-associated G-proteins, serine-threonine kinases, transcription factors and regulators of programmed cell death.

Growth factors play important roles in cell cycle control, and they signal a cell to either enter the G1 phase or bypass it. The right amount of growth factors should be produced at the right time, or else cell cycle control may be dysregulated. Examples of oncogene-encoded growth factors are *sis* oncogene-encoded platelet-derived growth factor (PDGF) B chain and *int-2* oncogene-encoded fibroblast growth factor (FGF)-related growth factor. Receptor-associated and non-receptor tyrosine kinases as well as G-proteins, G-protein-coupled receptors and serine-threonine kinases all encode products that are important in regulating the transduction of mitogenic signals in the cell, thereby regulating normal cell division. In most cases, these oncogenes encode mutant forms of the proteins so that they are not subject to the on-off regulation in response to mitogenic signals. In other words, the mitogenic signal is perpetually "on," resulting in uncontrolled cell proliferation.

Oncogenes, such as *src* and *abl*, encode protein tyrosine kinases that are membrane-associated, while *erbB*, *neu* and *fms* encode transmembrane kinases. An example of an oncogene encoding a GTP-binding GTPase is *ras*. Normal GTP-binding proteins (G-proteins), including *ras*, are important signal transducers. The active form binds GTP and transduces the mitogenic signal, whereas hydrolysis of GTP to GDP inactivates the protein and terminates the signal. Further activation involves exchange of GDP for GTP. Mutant forms of *ras* exist in an inactive GDP-bound form, resulting in a perpetual "on" mode of the mitogenic signal. Oncogenes encoding transcription factors also act by the same principle. The proto-oncogene products of *fos* (Fos) and *jun* (Jun)

form a heterodimer to produce the transcription factor *activator protein-1* (AP-1). Mutant AP-1 may act in a constitutive manner and does not require activation by its inducers, such as phorbol esters. Other oncogenes, such as *myc* and *myb*, also code for transcription factors that, when mutated, can cause aberrant transcriptional dysregulation.

The cell growth and division suppressor effects are also lost in mutant *p53* which is a tumor suppressor gene and whose product (p53 protein) is a transcription factor. The three-dimensional structure of p53 protein with its target DNA molecule defined the core domain and the amino acids involved in DNA binding (Cho *et al.*, 1994). These amino acids that are involved in DNA binding show the highest mutation rate in various cancers. This demonstrates how mutations in tumor suppressor genes can abrogate their tumor suppressor function by disrupting the transcriptional regulation of their target genes. Another role of p53 is to regulate apoptosis or programmed cell death by up-regulating the pro-apoptotic gene *Bax*. Mutant p53 cannot mediate apoptosis; thus cells with unrepaired DNA damage are prevented from undergoing apoptosis. Survival of these cells and their subsequent division may lead to the development of cancer cells.

Mutagenic chemicals that can disrupt the structure and/or expression of these proto-oncogenes can cause their oncogenic activation. For example, phorbol ester is a known activator of AP-1-mediated transcription. The procarcinogen 1,2 dimethylhydrazine has been reported to induce mutation in *ras* which was detectable in preneoplastic and neoplastic rat colonic mucosa (Jacoby *et al.*, 1991).

## **Radiation carcinogenesis**

### *Radiation dose and risk*

In the following discussion, radiation will refer to only ionizing radiation. A radiation dose to tissue is expressed as *absorbed energy per unit tissue mass*. Gray (Gy) is the unit of radiation dose and is quantified as 1 joule/kg. The older unit *rad* is still used and 1 rad = 0.01 Gy. Carcinogenic potential depends upon the absorbed dose (energy).

LET (linear energy transfer; *L*) is a measure of the rate at which energy (*E*) is deposited to the absorbing medium per unit distance (*l*) traversed by the radiation ( $L = dE/dl$ ; if the distance traversed is measured in mm, then  $L = \text{keV/mm}$ ). Consequently, high LET radiations (e.g.,  $\alpha$ -particles, neutrons, heavy ions, pions, also known as pi mesons) will deposit greater amount of energy than low LET radiations (e.g.,  $\gamma$ -rays, X-rays, electrons) in the absorbing media. Human skin can stop

$\alpha$ -particles from penetrating and reaching the internal organs. This is because  $\alpha$ -particles deposit most of their energy onto the skin and may damage the skin in that process. Both alpha and beta particles penetrate cell membranes more easily than they penetrate human skin. Therefore, ingesting or inhaling radioactive chemicals that can emit  $\alpha$ - or  $\beta$ -particles can pose serious threats to human health.

In the case of tissue exposure, the energy deposited by the radiation causes ionizations and the generation of free radicals, which cause macromolecular damage. Thus, high-LET radiations are more destructive to biological materials than low-LET radiations because at the same dose, low-LET radiations induce the same number of radicals more sparsely within a cell, whereas high-LET radiations transfer most of their energy to a small region of the cell. The localized DNA damage caused by dense ionizations from high-LET radiations is more difficult to repair than the diffuse DNA damage caused by the sparse ionizations from low-LET radiations.

Experimental studies with animals as well as epidemiological studies indicate that higher or continual radiation exposure increases the incidence of specific cancers. Increased incidence of lung cancer has been observed among uranium miners, fluorspar miners, zinc and iron ore miners (Adams and Cox, 1997). Gottlieb and Husen (1982) studied the incidence of lung cancer among American Indians. According to the authors, lung cancer has been a rare disease among the Indians of the southwestern United States, but the advent of uranium mining in the area has been associated with an increased incidence of lung cancer among Navajo uranium miners. The study centered on Navajo men with lung cancer who were admitted to the hospital from February 1965 to May 1979. Of a total of 17 patients with lung cancer, 16 were uranium miners. The cause of the lung cancers is the  $\alpha$ -particle radiation from the inhaled radon gas emanating from the radium present in the ore. Another well-documented example of radiation-induced cancer is the occurrence of osteosarcoma among workers in luminous dial watch factories. Workers used to lick the paint brush to maintain sharp edges and, in the process, consumed radium-226 and radium-228. The ingested radium deposited in the bone and was the source of short range  $\alpha$ -particles. A recent example of radiation-induced increases in thyroid cancer comes from the Chernobyl incident. The nuclear reactor explosion at Chernobyl in 1986 resulted in substantial contamination of some parts of Belarus, Ukraine and Bryansk. Immediately after the accident, these regions were contaminated with cesium-137 as well as various isotopes of iodine, including iodine-131 (Stsjazhko *et al.*, 1995). Cesium-137 is a very dangerous radioisotope to the environment because of its long-term effects. Its half-life of about 30 years ensures that it stays in the environment for a very long time. The

authors reported that of those who continued to live in the contaminated region and consume locally produced milk for the 3 months after the accident, most (about 85%) of the radiation dose to the thyroid was derived from iodine-131, while the remainder was from short-lived isotopes of iodine. The incidence of thyroid cancer among children under 15 was 30.6 per million during 1991–1994 as compared to 0.3 during 1981–1985. Another source of human data on carcinogenesis by ionizing radiation is from the A-bomb survivors from Hiroshima and Nagasaki. Data show that in the first 5–10 years after the exposure, the risk of leukemia increased rapidly but declined thereafter. The risk of solid tumors in many organs also increased significantly (Okey *et al.*, 1998).

### *Mechanism of radiation-induced carcinogenesis*

An absorbed dose of 1 Gy generates about  $2 \times 10^5$  ionizations within the mammalian cell. Approximately 1% of these ionizations occur in the DNA itself (Adams and Cox, 1997). Consequent DNA damage involves both single-strand and double-strand breaks, most of which are repaired within a few hours. Single-strand breaks, which are more frequently caused by low-LET radiations, are more readily repaired than double-strand breaks. UV radiation is known to distort the DNA strands by causing thymine dimers. In animals that have photolyase, thymine dimers are repaired by the direct repair mechanism that involves breaking of the bond causing the lesion. Humans do not have photolyase, so thymine dimers in humans are corrected by the nucleotide excision repair (NER) mechanism.

Ionizing radiation and oxidative stress are closely associated. Irradiated cells produce damaging reactive oxygen species (ROS), which can cause severe damage to cellular macromolecules including nuclear DNA (Spitz *et al.*, 2004; Wu *et al.*, 1999). A cell's oxidative status plays an important role not only at the time of radiation exposure, but also long after exposure. Irradiation may produce ROS for several minutes or even hours after exposure (Spitz *et al.*, 2004).

At the cytological level, an extension of radiation-induced DNA damage is chromosome breakage. Radiation can induce aberrant intra-chromosomal crossing over that involves one or both chromatids. Radiation can also induce non-disjunction of homologous chromosomes resulting in trisomy in the  $F_1$  offsprings, as well as other chromosomal aberrations, such as translocations and deletions (Adams and Cox, 1997). Chromosomal breaks have been shown to occur at a higher frequency in certain fragile sites. In other words, depending on the energy, radiation can cause increased genomic instability.

Unrepaired DNA strand/chromosomal breaks can lead to deletion or scrambling of gene sequences. The hypoxanthine–guanine phosphoribosyltransferase (HPRT) locus



on X-chromosome is known to undergo such aberrations following high radiation exposure (Thacker, 1986). If the target gene is an oncogene or a tumor suppressor gene, then radiation-induced mutations in oncogenes or inactivation of tumor suppressor genes may have serious consequences.

It should be emphasized that most of the molecular data on radiation-induced carcinogenesis have been obtained from laboratory studies, including *in vitro* studies. For humans, epidemiological studies from accidents and disasters involving ionizing radiation also demonstrate increased risk of cancer owing to high exposure to radiation. Nevertheless, little reliable information is currently available on the increased risk factors for high-LET radiations or about dose-rate effects of low-LET radiations (Okey *et al.*, 1998).

## EPIGENETIC BASIS OF CARCINOGENESIS

The mechanisms of carcinogenesis discussed above mostly involve changes in DNA sequence and/or integrity. However, carcinogenesis has an important epigenetic component as well. Epigenetics can be defined as heritable changes in gene expression that are not accompanied by changes in DNA sequence. Three main mechanisms of epigenetic regulation of gene expression are mediated by: (1) DNA methylation, (2) histone modifications and (3) small non-coding RNA, such as microRNA (miRNA).

Aberrant gene function and altered patterns of gene expression are key features of cancer. Growing evidence shows that acquired epigenetic abnormalities participate with genetic alterations to cause this dysregulation (Jones and Baylin, 2007). Proper coordination of temporal silencing and activation of gene expression is the key to the orchestration of normal life processes. Dysregulation of gene expression results in dysregulation of normal physiological processes, which, in turn, paves the way to the development of diseased states. Epigenetic changes can collaborate with genetic changes to cause the evolution of a cancer because they are mitotically heritable (Jones and Baylin, 2007).

The epigenetic basis of carcinogenesis is probably the most widely studied epigenetic basis of diseased state. Studies over the last 30 years or so have confirmed that the genome in a cancer cell/tissue is characterized by global (genome-wide) hypomethylation and site-specific promoter hypermethylation. Many of these epigenetic changes probably occur very early in cancer development and may contribute to cancer initiation (Jones and Baylin, 2007; Sharma *et al.*, 2010).

Global DNA hypomethylation basically has two effects: increasing genomic instability and activating growth promoting genes. Hypomethylation affects repeat sequences, leading to increased genomic instability by promoting chromosomal rearrangements. Additionally, hypomethylation of retrotransposons can result in their activation and translocation to other genomic regions, thus increasing genomic instability. DNA hypomethylation can also activate growth-promoting genes (proto-oncogenes), thereby inappropriately turning on mitogenic signals. DNA hypomethylation can also lead to the loss of imprinting (LOI). In contrast to hypomethylation, which increases genomic instability and activates proto-oncogenes, site-specific hypermethylation contributes to tumorigenesis by silencing tumor suppressor genes (Sharma *et al.*, 2010). Interestingly, methylation-mediated silencing of tumor suppressor genes provides one of the two hits advocated in Knudson's two-hit theory (Knudson, 1971).

Silencing of gene expression in carcinogenesis also involves global loss of transcription-activating histone modifications, such as acetylation and activating methylation. All known histone acetylations are transcription activating. In contrast, certain histone methylations are transcription activating, such as histone H4 lysine 20 methylation, whereas others are transcription repressing, such as histone H3 lysine 9 and lysine 27 methylation. Loss of histone acetylation is carried out by histone deacetylase (HDAC), which is often overexpressed in various types of cancer (Sharma *et al.*, 2010). In addition to the loss of transcription-activating modifications, cancer cells also have active transcriptional silencing modifications, such as histone H3 lysine 9 and lysine 27 methylation.

The third arm of epigenetic regulation, the miRNAs, also shows widespread dysregulation of expression in carcinogenesis. Because miRNAs are involved in transcriptional regulation, cell proliferation and apoptosis, dysregulation in their expression can promote tumorigenesis. Oncogenic miRNAs (oncomirs) target tumor suppressors and growth inhibitory pathways, and are often up-regulated in various types of cancer. Examples of oncomirs are miR-21 (targets the tumor suppressor "phosphatase and tensin homolog deleted on chromosome 10 (PTEN)," which is up-regulated in human glioblastoma; miR-155, which is up-regulated in breast, lung and several hematopoietic malignancies; miR-17-92 cluster, which targets pro-apoptotic gene *Bim*, is overexpressed in many different types of cancer. In contrast to oncomirs, tumor suppressor miRNAs target oncogenes and growth promoting pathways, and are often down-regulated in cancers. For example, miR-15 and miR-16 target the anti-apoptotic gene *BCL2* and are down-regulated in chronic lymphocytic leukemia; miR-127 targets *BCL6* and is down-regulated in prostate and bladder tumors (Sharma *et al.*, 2010).

Therefore, the normal cellular epigenetic landscape is significantly altered in cancer. The underlying mechanisms that initiate these global changes are yet to be fully understood, so are the causative versus correlative changes. Nevertheless, recent studies indicate that some changes occur very early in cancer development and may contribute to cancer initiation (Jones and Baylin, 2007; Sharma *et al.*, 2010).

## CLASSIFICATION OF CARCINOGENS

The most widely used systems for classifying carcinogens comes from the International Agency for Research on Cancer (IARC). The U.S. Environmental Protection Agency (EPA) has also developed a very similar classification scheme. In the past 30 years, IARC has evaluated about 900 likely candidates for their cancer-causing potential in humans. Most of the agents are of probable, possible or unknown risk. Only about 90 are classified as carcinogenic to humans. The IARC classification scheme is described below.

Known human carcinogen

**Group 1** – There is sufficient evidence of carcinogenicity in humans.

Probable human carcinogen

**Group 2A** – There is limited evidence of carcinogenicity in humans, but sufficient evidence of carcinogenicity in experimental animals, and strong evidence that carcinogenesis in experimental animals is mediated by a mechanism that also operates in humans.

Possible human carcinogen

**Group 2B** – There is limited evidence of carcinogenicity in humans, and less than sufficient evidence of carcinogenicity in experimental animals.

Not classifiable for human carcinogenicity

**Group 3** – The evidence of carcinogenicity is inadequate or limited in experimental animals.

Not likely to be a human carcinogen

**Group 4** – Not carcinogenic to humans.

## ASSAYS FOR CARCINOGENS

Tests most frequently used to determine carcinogenic activity include (1) long-term bioassays and (2) short-term assays. Recently, building of databases of known/suspected carcinogenic compounds has helped in the development of computer programs that can predict the carcinogenic potential of new compounds using quantitative structure–activity relationships (QSAR). These

programs provide additional tools when there is ambiguous data or insufficient data to infer safety. Some of the common tests under each category are discussed below.

### Long-term bioassays

The National Toxicology Program (NTP) rodent cancer bioassay evolved out of the National Cancer Institute (NCI) cancer bioassay protocol of the 1970s. Groups of 50 or more rodents are assigned to control or treatment groups. Test substances are given by intubation, dietary or drinking water consumption, or dermal or inhalation exposure. The route of delivery is determined based on the most likely route that humans are expected to be exposed through. Dosing starts at age 5–6 weeks and lasts for 2 years, at which point surviving animals receive a complete histopathologic examination (Bucher, 2002). In a classic lifetime tumor bioassay, the maximum tolerated dose (MTD) for a particular chemical is determined in a dose-range finding study; then doses close to the MTD are administered to male and female rats and mice for 2 years. A statistically significant, chemical-associated increase in tumor count is taken as evidence of carcinogenicity (Parsons and McKinzie, 2001). These assays are expensive and time consuming. Additionally, the use of MTD is debatable since the high doses of chemical used to develop tumors often lead to cytotoxic effects in animal tissues. This cytotoxicity may lead to cell proliferation and other promoter-like events. Nevertheless, the rodent tumor bioassay has been used for carcinogenicity testing for many years, primarily for four reasons. First, the rodent tumor bioassay fulfills an obvious need for an experimental system. Second, *in vivo* exposure and tumor development are considered more relevant endpoints for assessing human cancer risk than *in vitro* tests with bacterial or mammalian cells or non-tumor endpoints. Third, the rodent tumor bioassay has been used for many years without disastrous human health consequences, and, finally, at the present time there is no other “ideal” assay with which to replace the rodent tumor bioassay (Parsons and McKinzie, 2001).

### Short-term assays

These assays may be used to determine a chemical’s ability to cause *mutational events*, *chromosomal aberrations* or *DNA damage in vitro* or *in vivo*.

Two major assays used to detect *mutational events* are the *Ames test* for bacterial mutagenesis and the *mammalian cell gene mutation assays*. The Ames test was developed in the early 1970s by Bruce Ames at the University of California, Berkeley. The Ames method is based on inducing growth in genetically altered strains of *Salmonella typhimurium*. These strains need histidine

in order to grow. If the chemical agent under investigation is a mutagen, it should cause some of the bacteria to undergo mutations so that some of these bacteria (revertants) can grow without histidine, just like the wild-type bacteria (hence, reverse mutation test). To evaluate the effect of metabolism on the chemical, such tests are performed in the presence and absence of S9 fraction (post-mitochondrial supernatant fraction). The S9 fraction contains liver enzymes, generally from a rat treated with a broad-spectrum xenobiotic-metabolizing enzyme inducer, such as Arochlor 1254. There are several altered *Salmonella* strains used in the Ames test, such as TA97, TA98, TA100, TA1535 and TA1537; each varies in sensitivity to specific mutagens. The Ames test yields a number, such as the number of revertants (could be as high as several hundred thousands) per microgram of a pure chemical (mutagen).

Unlike the Ames test, which measures reverse mutation (from mutant back to wild type), the mammalian cell gene mutation assays measure forward mutations at a specific locus. Two of the well-studied loci are the thymidine kinase (TK) locus in mouse lymphoma cells and the hypoxanthine–guanine phosphoribosyl transferase (HPRT) locus in Chinese hamster ovary cells. To evaluate the effect of metabolism of the chemical, such tests are performed in the presence and absence of S9 fraction.

Cytogenetic analysis for *chromosomal aberrations* enables direct observation of various chromosomal aberrations. Cells are arrested in metaphase, examined microscopically and the chromosomal aberrations are enumerated. The cells used could be Chinese hamster ovary (CHO) cells, human peripheral blood lymphocytes or rat lymphocytes. An alternate cytogenetic approach is the observation and scoring of *micronuclei*. A micronucleus is formed during the metaphase/anaphase transition. It may arise from a lagging chromosome (*aneugenic* event resulting in chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (*clastogenic* event). Another assay for measuring chromosomal aberrations is the *sister chromatid exchange* (SCE) assay, which detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. Sister chromatid exchange means a reciprocal interchange of the two chromatid arms within a single chromosome. This exchange is visualized during metaphase. Detection of SCEs requires some means of differentially labeling sister chromatids, such as by the incorporation of bromodeoxyuridine (BrdU) into chromosomal DNA for two cell cycles. The most commonly used assays employ mammalian bone marrow cells or peripheral blood lymphocytes, often from rodent species. All these assays could be performed in the presence and absence of metabolic activation systems (S9).

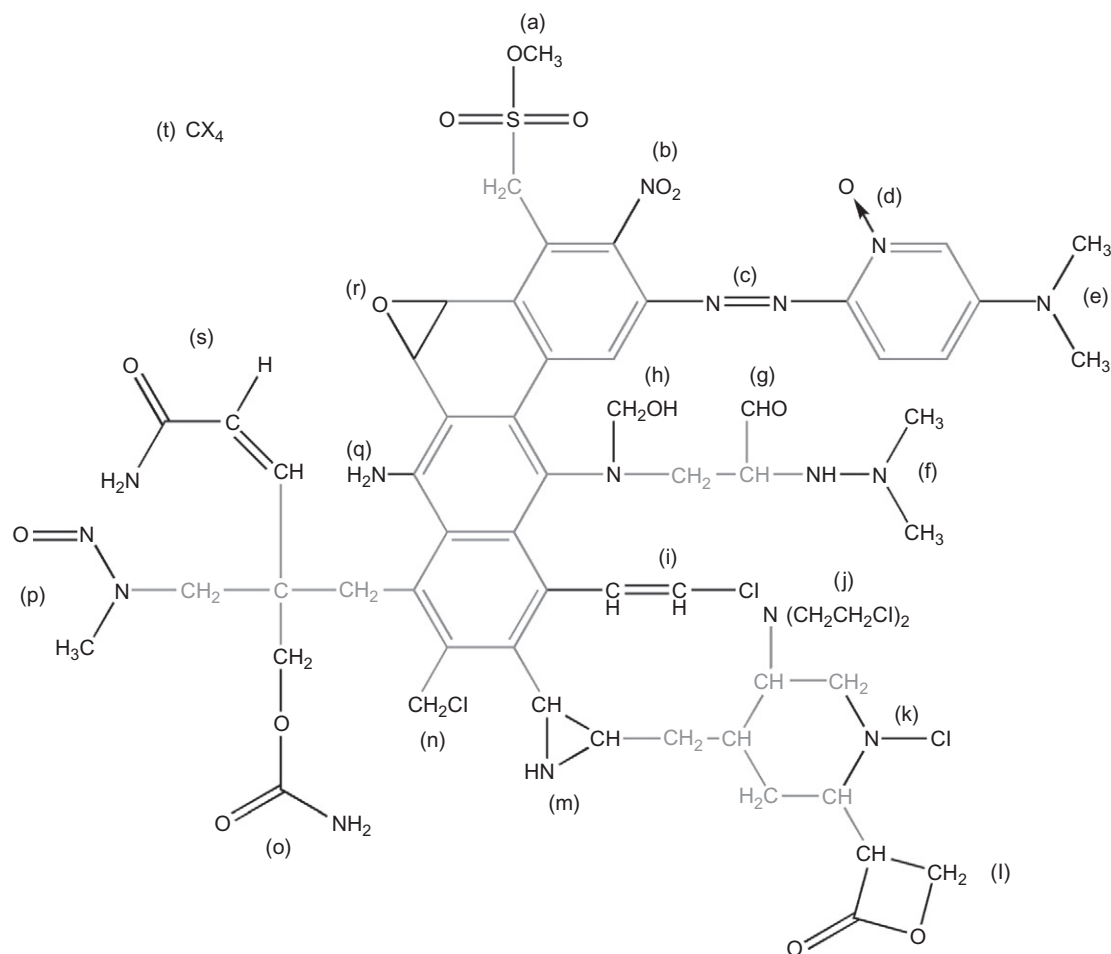
Detection of *DNA damage* can also be determined using the *comet assay*. The comet assay is also known as

the *single cell gel electrophoresis assay* and it is a very useful and sensitive technique for detecting DNA damage at the level of a single cell. The technique acquired its name from the comet-like shape of the DNA of the cells which can be seen under the microscope after the procedure. This technique was developed by Swedish researchers Östling and Johansson in 1984, and was later modified by Singh and coworkers in 1988 as the alkaline comet assay. The alkaline comet assay is much more sensitive than the original comet assay. In the comet assay, cells of interest are suspended in low-melting agarose and layered onto slides pre-coated with agarose. Cells are lysed under high salt concentration to release the damaged DNA. The DNA is subjected to unwinding under neutral/alkaline conditions to allow DNA supercoils to relax. Electrophoresis of this DNA under neutral or highly alkaline (pH > 13) conditions allows the broken ends to migrate forming the “comets.” After neutralization, staining is done using fluorescent DNA dyes (e.g., ethidium bromide, propidium iodide, Hoescht 33258, acridine orange, etc.) and the DNA is visualized under a fluorescent microscope. The slides are then scored by counting; 50–100 cells are counted per sample, either by manual counting or using specific software. Both qualitative and quantitative assessment of DNA damage is carried out. The alkaline comet assay enables the detection of single-strand DNA breaks in individual cells. Therefore, the alkaline comet assay can measure low levels of DNA strand breaks.

The ability of a genotoxic agent to induce DNA lesions can also be investigated by measuring the *unscheduled DNA synthesis* (UDS). The assay measures DNA repair synthesis after excision and removal of a stretch of DNA containing the lesion induced by chemical and physical agents. The assay is based on the incorporation of tritium-labeled thymidine into the DNA of mammalian cells that are not in the S phase (DNA synthesis phase) of the cell cycle. The uptake of tritium-labeled thymidine can be determined by autoradiography or by liquid scintillation counting of DNA from the treated cells.

### Structure–activity relationships and carcinogenicity

Over 70,000 chemicals are used commercially and more than 100,000 naturally occurring chemicals have been identified (Cramer *et al.*, 1978). It would be virtually impossible, impractical, economically unfeasible and not sensible to test all these compounds for potential carcinogenicity. This then begs the question – how do scientists (toxicologists, food technologists, drug developers, regulatory scientists, etc.) make decisions as to the safety of chemicals for addition to food, as drugs, for industrial use and/or for prioritizing those that do require additional, extensive toxicity testing?



**FIGURE 26.8** Hypothetical chemical structure highlighting (in black) the Ashby and Tennant structural alerts for carcinogenicity (Ashby and Tennant, 1989). (a) Alkyl ester of either phosphonic or sulphonic acids, (b) aromatic nitro groups, (c) aromatic azo groups, by virtue of their reduction to an aromatic amine, (d) aromatic ring *N*-oxides, (e) aromatic mono- and di-alkylamino groups, (f) alkyl hydrazines, (g) alkyl aldehydes, (h) *N*-methylol derivatives, (i) monohaloalkenes, (j) a large family of *N* and *S* mustards ( $\beta$ -haloethyl), (k) *N*-chloramines (substructure has not been associated with carcinogenicity, but potent genotoxic activity has been reported), (l) propiolactones and propiosultones, (m) aromatic and aliphatic aziridiny derivatives, (n) both aromatic and aliphatic substituted primary alkyl halides, (o) derivatives of urethane (carbamates), (p) alkyl *N*-nitrosamines, (q) aromatic amines, their *N*-hydroxy derivatives and the derived esters, (r) aliphatic epoxides and aromatic oxides, (s)  $\alpha,\beta$ -unsaturated carbonyls, (t) halogenated methanes ( $X = \text{H, Cl, Br, I}$  in any combination).

One of the ways toxicologists have prioritized the study of chemicals is through the use of structure–activity relationship analysis (SAR). Structure–activity relationships can be divided into two classes: (1) qualitative relationships and (2) quantitative relationships.

#### Qualitative structure–activity relationships

A qualitative structure–activity relationship is an association between a molecular (sub)structure and the presence or absence of a biological activity, or the capacity to modulate a biological activity imparted by another substructure. A substructure associated with the presence of a biological activity is sometimes called a structural alert. Therefore, chemicals with similar chemical structures and physicochemical properties should exhibit similar

biological activity whether it is a specific toxic effect or a pharmacological activity.

Qualitative SAR analyses rely upon a finite knowledge base of compounds that have been previously tested and the researcher's ability to identify key structural similarities between the compound of interest and compounds in the previously established knowledge bases. A variety of classification schemes, such as those detailed in Cramer *et al.* (1978), Cheeseman *et al.* (1999) and FDA's Red-book (Rulis *et al.*, 1984), have been developed that exploit these relationships, most of which are based on classifying either mutagens or carcinogens into broad categories. The Ashby and Tennant classification scheme (Ashby and Tennant, 1989) for structural alerts (Figure 26.8) is probably one of the simplest approaches to identifying potential carcinogenic activity of a compound



**TABLE 26.2** A list of Ashby and Tennant structural alerts and functional-group list compiled by Munro (Me = —CH<sub>3</sub>, E = —CH<sub>2</sub>CH<sub>3</sub>)

**Aryl and heterocyclic ring substituted amino- and nitro- derivatives (Ashby AA class)**

Bioactivation to nitrenium ions (Ar—N<sup>+</sup>—H) by N-hydroxylation to arylhydroxylamines (Ar—N(OH)R)  
 1°- and 2°-amines [Ar—NHR, where R may be —H, —Me, —Et or activated Me or Et] (examples mostly 1°-amines and include hydrochloride salts)  
 3°-amines [Ar—NR<sup>1</sup>R<sup>2</sup>, where R<sup>1</sup> and R<sup>2</sup> = —Me, —Et] [few examples mostly di-Me]  
 2°-acetamides and -formamides [Ar—NHCOR, where R = —H, —Me or activated Me] (examples mostly R=NHCOMe)  
 nitroarenes [Ar—NO<sub>2</sub>] (reduction to nitroarene; many examples)  
 nitrosoarenes [Ar—N=O] (reduction to arylhydroxylamine)  
 arylhydroxylamines [Ar—N(OH)R]

**Nitroso compounds (Ashby NO class)**

Bioactivation to carbonium (R<sub>2</sub>CH<sup>+</sup>) or diazonium ions (RN<sup>+</sup>≡N) by oxygenation at α-C to diazohydroxides (R-N=N—OH). Substrate must possess —H, —OH on α-C or α-C=O relative to nitrogen.  
 N-nitroso-N-dialkylamines [R<sup>1</sup>R<sup>2</sup>N—N=O, most examples are from this class]  
 N-nitroso-N-alkylamides [R<sup>1</sup>R<sup>2</sup>N—N=O, where R<sup>2</sup> = COR<sup>3</sup>, R<sup>3</sup> = alkyl or aryl]  
 N-nitroso-N-alkylureas [R<sup>1</sup>R<sup>2</sup>N—N=O, where R<sup>2</sup> = CONR<sup>3</sup>R<sup>4</sup>, R<sup>3</sup> = H, R<sup>4</sup> = alkyl or aryl]  
 N-nitroso-N-alkylcarbamates (aka -urethanes) [R<sup>1</sup>R<sup>2</sup>N—N=O, where R<sup>2</sup> = CO<sub>2</sub>R<sup>3</sup>, R<sup>3</sup> = alkyl or aryl]  
 N-nitroso-N-alkylnitriles [R<sup>1</sup>R<sup>2</sup>N—N=O, R<sup>2</sup> = CN]  
 N-nitroso-N-hydroxylamines [R-N(OH)NO]

**Hydrazo derivatives (Ashby NZN class)**

Bioactivation to carbonium (R<sub>2</sub>CH<sup>+</sup>) or diazonium ions (RN<sup>+</sup>≡N) by oxygenation at α-C. R groups may be alkyl or aryl, but one must possess —H, —OH on α-C or α-C=O relative to nitrogen  
 hydrazines [R<sup>1</sup>R<sup>2</sup>N—NR<sup>3</sup>R<sup>4</sup>] (oxidation to azo then azoxy derivative; examples mostly terminal R<sub>2</sub>N—NH<sub>2</sub>)  
 azoxy alkane [R<sup>1</sup>-N<sup>+</sup>(O<sup>-</sup>)=N-R<sup>2</sup>, R is C<sub>4</sub> or less]

**Natural electrophiles (Ashby ALK class)**

Aliphatic halides [R-CH<sub>2</sub>X, where R is ≤ C<sub>4</sub> and contains activating groups; not more than one—X per C]  
 Benzylic halides [Ar—CH<sub>2</sub>X, where X = Cl, Br, I]  
 Oxiranes and aziridines [3-membered O- and N-containing rings]  
 Propiolactones [4-membered ring containing—CO<sub>2</sub>-]  
 Alkyl esters of sulfonic [RSO<sub>2</sub>OR<sup>1</sup>] and sulfuric [ROSO<sub>2</sub>OR<sup>1</sup>] acids (where R<sup>1</sup> = Me or Et)  
 Alkyl esters of phosphonic [RP(=O)(OR<sup>1</sup>)<sub>2</sub>] and phosphoric [(RO)<sub>2</sub>P(=O)OR<sup>1</sup>] acids (where R<sup>1</sup> = Me or Et)  
 Mixed alkyl esters of phosphoric acid [(RO)<sub>2</sub>P(=S)OR<sup>1</sup>, where R<sup>1</sup> = Me or Et]  
 Haloethylamines [—NCH<sub>2</sub>CH<sub>2</sub>X]  
 Haloalkylethers [ethyl (—OCH<sub>2</sub>CH<sub>2</sub>X) and methyl (—OCH<sub>2</sub>X)]  
 α-Halocarbonyl [R(C=O)CH<sub>2</sub>X] or α-halohydroxy [R(CHOH)CH<sub>2</sub>X]  
 Haloamines [R<sub>2</sub>N—X]  
 α,β-Unsaturated carbonyls [R<sub>2</sub>C=C—C(=O)R, where R is an aldehyde, ketone, ester, amide group]  
 Allylic halides and alkoxides [R<sub>2</sub>C=C—CH<sub>2</sub>X, where X = Cl, Br, I or OR]

**Other alerting groups (Ashby SA+ class)**

Halogenated methanes [CH<sub>m</sub>X<sub>n</sub>, where m + n = 4]  
 Vinyl halides [R<sup>1</sup>R<sup>2</sup>C=CHX, where X = Cl, Br or I]  
 Polycyclic aromatic hydrocarbons  
 Isocyanate [R-N=C=O]  
 Isothiocyanate [R-N=C=S]  
 Azoarenes [R<sup>1</sup>-N=N—R<sup>2</sup>, R<sup>1</sup> and R<sup>2</sup> are aryl] (-SO<sub>3</sub>H group on both rings non-alerting)

Source: Bailey *et al.* (2005)

that has unknown toxicity. This classification scheme correlates a structural alert (a fragment of a chemical's structure that may participate in an electrophilic attack on DNA) with a compound's carcinogenic potential. These alerts can be used in the evaluation of a chemical to give a qualitative sense of the compound's likelihood of being carcinogenic. A list of structural alerts proposed by Ashby and Tennant (Ashby and Tennant, 1989) and functional groups cited by Munro (Munro *et al.*, 1996) are summarized in Table 26.2.

In addition to using classification schemes to identify compounds for potential concerns, there are a number of structurally searchable databases that allow one to identify close structural analogs of the compound in question. Publicly available examples are the National Library of Medicine's (NLM) ChemIDPlus (NLM, 2005) and EPA's Distributed Structure-Searchable Toxicity (DSSTox) Public Database Network (EPA, 2005). Several commercial databases of toxicity information are also available; two examples are Leadscope Enterprise and Lhasa Limited's Vitic

Nexus. The data from these analogs may be used to extrapolate a numerical risk value for a potential toxic effect.

### Quantitative structure–activity relationships

Quantitative SAR analysis (QSAR) is a quantitative relationship between a biological activity (e.g., toxicity) and one or more descriptors that are used to predict the activity. In other words, QSAR utilizes the existing scientific data to make decisions about the potential carcinogenicity of new and/or untested chemicals using predictive models based on some type of algorithm (e.g., relating structural topology, chemical bond types, bioavailability, solubility, etc. to some toxic endpoint – genotoxicity, carcinogenicity, etc.) powered by computer software.

There are several commercially available predictive toxicity programs currently available. These programs can be divided into two general categories: (1) human expert/rule-based programs such as OncoLogic and Derek Nexus and (2) human expert/statistical/correlative programs such as Leadscape Model Applier, TOPKAT and MCASE-MC4PC. Detailed information on these and other QSAR software packages is readily obtained on the World Wide Web.

An example of a human expert/rule-based program is OncoLogic, which predicts carcinogenicity based on the chemical structure of a substance (Woo *et al.*, 1995). Analysis is done by applying a decision tree approach that incorporates the knowledge of mechanisms of action and human epidemiological studies. OncoLogic contains four carcinogenicity modules focused on non-dietary fibers, metals or organometallics, polymers and organics. The output consists of a justification of the potential carcinogenicity, or lack thereof, using a tiered level of concern (low, marginal, low-moderate, moderate, high moderate or high) with identification of the chemicals used in the decision tree analysis that led to the conclusions.

An example of a statistical/correlative program is MCASE-MC4PC (MultiCase, 2005), which has several predictive modules, including mutagenicity, carcinogenicity and teratogenicity. MultiCase uses an algorithm to identify all possible 2–10 atom fragments for each discrete chemical structure within a diverse training set of substances. The program then identifies fragments (structural alerts or biophores) that are primarily associated with the biological activity (e.g., carcinogenicity) of the training set and modulators of the activity, such as bioavailability and conformational comparisons. New substances with unknown toxicity are screened by entering the structure of the untested substance using a structural drawing program. The molecule in question is reduced to all possible 2–10 atom fragments and these fragments are compared to the list of structural alerts (biophores) and modulators identified within the training set. The output consists of scalar units, referred to as

CASE units, in increasing activity values ranging from 10 to 80. A case rating of 10–19 is considered non-toxic, 20–29 are marginal (equivocal, weak or inconsistent findings), 30–49 are moderately potent toxins (*trans*-gender, single site tumors) and 50–80 are potent toxins (*trans*-species, *trans*-gender, multiple site tumors in rodents). The biophores identified by the program are subjected to a set of human expert rules that take into account specificity, positive predictivity, false positives and coverage to arrive at a decision as to whether the test molecule is carcinogenic, the level of confidence with the prediction and whether the program-generated CASE-unit score is appropriate.

In the past, (Q)SAR models were typically used individually. However, more recently there has been a movement to use multiple (Q)SAR models in conjunction with each other to make toxicity predictions. The use of multiple (Q)SAR models, both statistical and rule-based predictive software, can be used to decrease false-positive and false-negative predictions. However, this approach presents a unique problem; how do you deal with the multiple predictions from these packages? You can take the approach of one positive prediction among the set means the compound is positive for the tested endpoint. This is a very conservative approach that could lead to additional, unnecessary testing. Alternatively, you could use a consensus approach (Matthews *et al.*, 2008) where the compound is only called positive or negative if all of the models result in the same prediction. This approach may reduce the concerns expressed regarding the false-positive and false-negative rates of the models since the models are all in agreement. However, a significant challenge still remains; what do you do with a compound where the QSAR models' predictions are conflicting? An approach to these conflicting predictions is that they are evaluated to ensure that they are valid and are weighed against predictions from rule-based systems such as Derek Nexus, Oncologic and, when possible, information gathered from commercial and/or publicly available toxicity databases, thus creating a set of "checks and balances" by using multiple models that evaluate the chemicals in different ways, such as: biophores, whole molecule properties, fingerprints, as well as rule-based predictions. Using these predictions in conjunction with toxicity data on the compound, or its structural analogs, a weight-of-evidence approach can be used to predict the potential toxicity or provide sufficient evidence to support additional data/testing needs. This approach of using multiple (Q)SAR models has the added benefit that if the chemical in question is not covered by one (Q)SAR model, it will most likely be covered by one or more of the other models.

Predictive software continues to be refined and its concordance, specificity and predictivity are approaching

the 90+ percent range. As long-term testing becomes increasingly expensive and controversial (animal rights activism, etc.), look for these QSAR software packages to play an increasing and more prominent role in the safety assessment of new chemical substances.

The accumulated knowledge of the metabolism, chemical reactivity, exposure, structure–activity relationships and other relevant information on chemicals, when scientifically and systematically woven together, can allow us to make expert judgments as to the potential risks due to exposure to chemicals in food, air and water. These judgments can be used to identify those compounds that merit further toxicity testing and those not worth pursuing. All schemes used to make these decisions must be thoroughly tested, be transparent and evoke confidence in the results both in the public and the scientific communities.

## CONCLUSIONS

After more than three decades since President Nixon declared the “War on Cancer” with the enactment of the National Cancer Act, the war still continues. Routine check-up has been effective in the early diagnosis and cure of certain forms of cancer, such as colon cancer and breast cancer. Inventions in modern medicine have further added to better prognosis. Nevertheless, cancer largely remains an incurable disease unless it is detected early.

In spite of significant advances in our knowledge on the molecular mechanisms of carcinogenesis, scientists seem to have a long way to go before all these advances in knowledge could be translated into effective and curative therapy. In the meantime, more work is needed to understand certain aspects of carcinogenesis, such as the mechanisms of action of non-genotoxic carcinogens. However, the accumulated knowledge on various aspects of carcinogenesis has definitely helped scientists make appropriate risk assessment and health policy recommendations. Finally, recent progress in the science of genomics has provided scientists with renewed hope that further breakthroughs in cancer detection and therapy are not far away, allowing us to effectively control the disease.

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# Oxidative stress and chemical toxicity

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## INTRODUCTION

Oxidative stress is characterized by the presence of an excess of oxidants caused by an imbalance between the formation and neutralization of reactive oxygen species (ROS) (Behl, 1999). Oxidative stress can lead to the formation of chain reaction byproducts, which create a potentially unstable cellular environment by causing massive cellular injury and extensive damage to the lipids, proteins and biological macromolecules. Oxidative stress has also been linked to tissue damage, advancing age associated dysfunctions and a broad spectrum of degenerative diseases including atherosclerosis, arthritis, cardiovascular dysfunctions, diabetes, metabolic syndrome and chronic fatigue syndrome (Javadov *et al.*, 2011; Maicas *et al.*, 2011).

Free radicals or oxidants are highly reactive, unstable molecules that have an unpaired electron in their outermost electron orbit which reacts with or oxidizes various cellular components and biological macromolecules including proteins, DNA, RNA, lipids, fatty acids and advanced glycation end products, i.e., carbonyls (Stohs and Bagchi, 1995). These reactions between cellular components and free radicals lead to DNA single- and double-strand breaks, DNA fragmentation, lipid peroxidation, protein oxidation products, mitochondrial malfunction, cell membrane damage, lactate dehydrogenase (LDH) leakage and eventually programmed cell death (apoptosis) and unprogrammed cell death (necrosis). Examples of free radicals or oxidizing molecules are hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), singlet oxygen, superoxide anion ( $\bullet\text{O}_2^-$ ), hydroxyl radical ( $\bullet\text{OH}$ ), peroxy radical ( $\text{ROO}\bullet$ ), reactive nitrogen oxides and peroxynitrite ( $\text{ONOO}^-$ ) (Table 27.1).

The steady-state formation of free radicals is normally balanced by a similar rate of consumption of antioxidants. Various pathologic processes disrupt this balance by increasing the formation of free radicals in proportion to the available antioxidants and antioxidant enzymes. Enhanced formation of free radicals induces a compromised effect on the body's immune system leading to inflammation, ischemia-reperfusion injury of different organs, apoptosis, necrosis and altered gene expression. Enhanced formation of free radicals and oxidative stress, and inadequate antioxidant activity are associated with a broad spectrum of disease states such as cardiovascular dysfunctions, chronic fatigue syndrome, hepatitis, cancer, autoimmune disorders, HIV and AIDS, cancer and diverse neurodegenerative diseases (Stohs, 1995; Stohs and Bagchi, 1995). This chapter describes chemical-induced oxidative stress and its consequences on animal health.

## OXIDATIVE STRESSORS: HEAVY METALS AND ENVIRONMENTAL TOXICANTS

Numerous evidence suggests that exposure to structurally diverse environmental toxicants, including heavy metals, polyhalogenated or polycyclic aromatic hydrocarbons, pesticides and their carcinogenic counterparts, and mycotoxins may involve a common cascade of events which includes the production of ROS and oxidative stress leading to lipid peroxidation (Bagchi *et al.* 1998a, 2002; Hussein and Brasel, 2001), DNA single-strand breaks and fragmentation (Stohs *et al.*, 1997),

TABLE 27.1 Structurally diverse oxygen free radicals involved in oxidative stress and chemical toxicity

Oxidant	Description
$\bullet\text{O}_2^-$ , superoxide anion	One-electron reduction state of $\text{O}_2$ , formed in many autooxidation reactions and by the electron transport chain. Rather unreactive but can release $\text{Fe}^{2+}$ from iron-sulfur proteins and ferritin. Undergoes dismutation to form $\text{H}_2\text{O}_2$ spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed $\bullet\text{OH}$ formation.
$\text{H}_2\text{O}_2$ , hydrogen peroxide	Two-electron reduction state, formed by dismutation of $\bullet\text{O}_2^-$ or by direct reduction of $\text{O}_2$ . Lipid soluble and thus able to diffuse across membranes.
$\bullet\text{OH}$ , hydroxyl radical	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components
$\text{ROOH}$ , organic hydroperoxide	Formed by radical reactions with cellular components such as lipids and nucleobases.
$\text{RO}\bullet$ , alkoxy and $\text{ROO}\bullet$ , peroxy radicals	Oxygen-centered organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
$\text{HOCl}$ , hypochlorous acid	Formed from $\text{H}_2\text{O}_2$ by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
$\text{ONOO}^-$ , peroxynitrite	Formed in a rapid reaction between $\bullet\text{O}_2^-$ and $\text{NO}\bullet$ . Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.

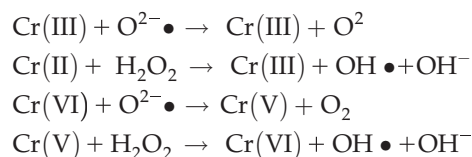
membrane damage with decreased membrane fluidity (Bagchi *et al.*, 1992), apoptosis (Stohs, 1995), glutathione depletion (Bagchi *et al.*, 1996a), altered calcium homeostasis (Bagchi *et al.*, 1997, 1998a, 1998b), enhanced release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), induction of stress/heat shock protein 90 (Von Burg and Lui, 1993), stimulation of oncogene expression, and inhibition of tumor suppressor genes (Schwarz *et al.*, 1995).

## Chromium, lead, cadmium and other heavy metals

### Chromium

Chromium exists predominantly in two valence states: Cr(III) and Cr(VI). Cr(III) is an essential micronutrient, while Cr(VI) is carcinogenic. Cr(VI) is a highly reactive transition metal widely known to cause allergic dermatitis as well as toxic and carcinogenic effects (Von Burg and Lui, 1993; Goyer 1996). Chromate,  $(\text{CrO}_4)^{2-}$ , is the most dominant form of Cr(VI) in neutral aqueous solutions and can readily cross cellular membranes via non-specific anion carriers (Danielsson *et al.*, 1982). The ability of Cr(VI) to induce an oxidative stress is well known. Von Burg and Lui (1993) and Barceloux (1999) summarized the acute and chronic toxicity, neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, immunotoxicity and general environmental toxicity of chromium. Chromium (VI)-induced internucleosomal DNA fragmentation, inhibition of macromolecular synthesis and apoptosis have been demonstrated in cultured Chinese hamster ovary cells, and low-dose exposure to chromium has been shown to potentiate cell proliferation and apoptosis (Von Burg and Lui, 1993; Manning *et al.*, 1994; Barceloux 1999). Vitamin E,

glutathione and oxygen scavengers can decrease chromate-induced cytotoxicity, lipid peroxidation and DNA damage, with vitamin E exhibiting the greatest protective effect (Sugiyama, 1991). Both forms of chromium undergo redox cycling (Stohs and Bagchi, 1995). Various investigators have examined the mechanism of DNA cleavage by Cr(VI). The results indicate that Cr(VI) complexes are produced in the reduction of Cr(VI) by cellular reductants such as NADPH that react with hydrogen peroxide to generate hydroxyl radicals (Shi and Dalal, 1989). The hydroxyl radicals are believed to be the initiators of the primary events in Cr(VI) cytotoxicity and are responsible for causing DNA strand breaks. The reactions involved in the redox cycling of Cr(III) and Cr(VI) are:



Although Cr(III) is much less nontoxic compared with Cr(VI), Cr(III) can be reduced to Cr(II) by biological reductants such as NADH and L-cysteine (Ozawa *et al.*, 1993). The newly formed Cr(II) reacts with hydrogen peroxide to produce hydroxyl radical, which is presumably responsible for tissue-damaging effects including the production of DNA damage. In summary, both Cr(VI) and Cr(III) are biologically active oxidation states of chromium, although Cr(VI) is much more toxic and produces greater oxidative stress and carcinogenesis. Both oxidation states of chromium are involved in redox cycling with the production of ROS.

Investigations have shown that sodium dichromate (Cr(VI)) more effectively induces the formation of ROS and

causes oxidative tissue and DNA damage compared with Cr(III) (Manning *et al.*, 1994; Bagchi *et al.*, 1995a). The effect of an oral low dose (0.05 LD<sub>50</sub>) of Cr(VI) (2.5 mg/kg/d) on brain mitochondrial and microsomal lipid peroxidation, excretion of urinary lipid metabolites, and brain nuclear DNA single-strand breaks in Sprague-Dawley rats has shown that maximum increases in these parameters occur between 60 and 75 days of treatment. The results of these experiments clearly indicate that low-dose subchronic administration of Cr(VI) induces oxidative stress, resulting in tissue damaging effects that may contribute to the toxicity and carcinogenicity of Cr(VI) (Bagchi *et al.*, 2002).

The *p53* tumor suppressor gene plays a major role in the regulation of cellular stress response including oxidative stress, in part through the transcriptional activation of genes involved in cell cycle control, DNA repair and apoptosis (Amundson *et al.*, 1998). Many factors have been shown to contribute to control the activation of *p53*, and the downstream response to *p53* activation may vary depending on the cellular environment or other modifying factors in the cell, including exposure to diverse xenobiotics. Because *p53* is activated in response to DNA damage and different signaling mechanisms, the role of the *p53* gene was assessed to unveil the mechanism of structurally diverse xenobiotics-induced oxidative stress and toxicity in the brain tissues (Bagchi *et al.*, 2000). Measured biomarkers included superoxide anion production (cytochrome *c* reduction), DNA

fragmentation and lipid peroxidation (TBARS). The levels of these parameters were not significantly different in the brains of untreated C57BL/6NTac and C57BL/6TSG *p53*-deficient mice. A comparative study was conducted using different types of environmental stressors including TCDD, endrin, naphthalene and Cr(VI) in *p53*-deficient mice. Treatment of C57BL/6NTac mice with a 0.01 LD<sub>50</sub> dose of TCDD, endrin, naphthalene and Cr(VI) increased cytochrome *c* reduction by 1.5- to 1.6-fold in brain tissues as compared to the control animals, although under the same conditions, approximately 1.7- to 2.4-fold increases in cytochrome *c* reduction were observed in brain tissues of *p53*-deficient mice (Table 27.2). Similar results were obtained in brain lipid peroxidation (Table 27.3) and DNA fragmentation (Table 27.4) (Bagchi *et al.*, 2000).

Approximately 2.5-, 3.0-, 3.4- and 2.5-fold increases in cytochrome *c* reduction were observed in brain tissues of C57BL/6NTac mice after treatment with 0.10 LD<sub>50</sub> doses of TCDD, endrin, naphthalene and Cr(VI) compared to corresponding values in untreated control animals. Under the same conditions, approximately 4.0-, 6.9-, 5.1- and 3.7-fold increases in cytochrome *c* reduction were observed in brain tissues of TSG-*p53* mice compared to control animals. In comparison, at a 0.10 LD<sub>50</sub> dose, TCDD, endrin, naphthalene and Cr(VI) induced 1.6-, 2.3-, 1.5- and 1.5-fold greater increases in cytochrome *c* reduction in brain tissues of *p53*-deficient mice than in

TABLE 27.2 Cytochrome *c* reduction in brain tissue (nmoles reduced/15 min/mg of protein)

Treatment	0.50 LD <sub>50</sub>		0.10 LD <sub>50</sub>		0.01 LD <sub>50</sub>	
	C57BL/6NTac	<i>p53</i> -deficient	C57BL/6NTac	<i>p53</i> -deficient	C57BL/6NTac	<i>p53</i> -deficient
Control	5.78 ± 0.41 <sup>a</sup>	7.34 ± 0.65 <sup>a</sup>	5.78 ± 0.41 <sup>a</sup>	7.34 ± 0.65 <sup>a</sup>	5.78 ± 0.41 <sup>a</sup>	7.34 ± 0.65 <sup>a</sup>
TCDD	33.07 ± 5.22 <sup>b</sup>	59.60 ± 11.71 <sup>a</sup>	14.21 ± 1.80 <sup>b</sup>	29.38 ± 4.74 <sup>b</sup>	8.66 ± 1.25 <sup>b</sup>	12.31 ± 1.68 <sup>b</sup>
Endrin	31.28 ± 6.64 <sup>b</sup>	74.12 ± 16.27 <sup>b</sup>	17.55 ± 2.19 <sup>b,c</sup>	50.32 ± 9.07 <sup>c</sup>	9.29 ± 1.05 <sup>b</sup>	16.67 ± 1.79 <sup>c</sup>
Naphthalene	30.61 ± 6.45 <sup>b</sup>	70.33 ± 8.29 <sup>b</sup>	19.81 ± 2.40 <sup>c</sup>	37.58 ± 6.44 <sup>b</sup>	9.05 ± 1.35 <sup>b</sup>	17.30 ± 3.41 <sup>c</sup>
Cr(VI)	23.83 ± 5.56 <sup>b</sup>	56.80 ± 8.94 <sup>b</sup>	14.57 ± 2.08 <sup>b</sup>	26.94 ± 4.58 <sup>b</sup>	9.39 ± 1.22 <sup>b</sup>	12.85 ± 1.76 <sup>b</sup>

Cytochrome *c* reduction as an index of superoxide anion production was determined in brain tissue homogenates of C57BL/6NTac and *p53*-deficient mice 24 h after treatment with a 0, 0.01, 0.10 or 0.50 LD<sub>50</sub> dose of the xenobiotics. Each value is the mean ± SD of four experiments. <sup>a, b, c</sup> Values within a column with non-identical superscript symbols are significantly different (*p* < 0.05) from each other.

TABLE 27.3 Lipid peroxidation in brain tissue (nmol MDA/mg protein)

Treatment	0.50 LD <sub>50</sub>		0.10 LD <sub>50</sub>		0.01 LD <sub>50</sub>	
	C57BL/6NTac	<i>p53</i> -deficient	C57BL/6NTac	<i>p53</i> -deficient	C57BL/6NTac	<i>p53</i> -deficient
Control	1.69 ± 0.19 <sup>a</sup>	2.02 ± 0.24 <sup>a</sup>	1.69 ± 0.19 <sup>a</sup>	2.02 ± 0.24 <sup>a</sup>	1.69 ± 0.19 <sup>a</sup>	2.02 ± 0.24 <sup>a</sup>
TCDD	5.36 ± 0.50 <sup>b</sup>	11.53 ± 1.82 <sup>b</sup>	3.32 ± 0.29 <sup>b</sup>	7.56 ± 0.80 <sup>b</sup>	1.74 ± 0.21 <sup>a</sup>	2.53 ± 0.19 <sup>b</sup>
Endrin	6.68 ± 0.74 <sup>c</sup>	19.16 ± 2.32 <sup>c</sup>	2.90 ± 0.41 <sup>b</sup>	5.52 ± 0.79 <sup>c</sup>	2.07 ± 0.31 <sup>a</sup>	2.44 ± 0.36 <sup>a</sup>
Naphthalene	6.52 ± 0.75 <sup>c</sup>	23.43 ± 4.19 <sup>c</sup>	1.99 ± 0.30 <sup>a</sup>	4.25 ± 0.50 <sup>d</sup>	1.83 ± 0.47 <sup>a</sup>	2.20 ± 0.17 <sup>a</sup>
Cr(VI)	5.88 ± 0.81 <sup>b,c</sup>	14.71 ± 1.69 <sup>d</sup>	2.84 ± 0.42 <sup>b</sup>	4.79 ± 0.33 <sup>d</sup>	1.87 ± 0.32 <sup>a</sup>	2.92 ± 0.36 <sup>b</sup>

Lipid peroxidation was based on the formulation of TBARS. C57BL/6NTac and *p53*-deficient mice were killed 24 h after treatment with a 0, 0.01, 0.10 or 0.50 LD<sub>50</sub> dose of xenobiotics. Each value is the mean ± SD of four experiments. MDA was used as the standard. <sup>a-d</sup> Values within a column with non-identical superscript symbols are significantly different (*p* < 0.05) from each other.

TABLE 27.4 DNA fragmentation in brain tissue (exposed as a percentage of total DNA in supernatant fraction)

Treatment	0.50 LD <sub>50</sub>		0.10 LD <sub>50</sub>		0.01 LD <sub>50</sub>	
	C57BL/6NTac	p53-deficient	C57BL/6NTac	p53-deficient	C57BL/6NTac	p53-deficient
Control	2.96 ± 0.40 <sup>a</sup>	2.76 ± 0.24 <sup>a</sup>	2.96 ± 0.40 <sup>a</sup>	2.76 ± 0.24 <sup>a</sup>	2.96 ± 0.40 <sup>a</sup>	2.76 ± 0.24 <sup>a</sup>
TCDD	7.12 ± 0.63 <sup>b</sup>	11.57 ± 1.50 <sup>b,c</sup>	4.01 ± 0.29 <sup>b</sup>	6.48 ± 0.75 <sup>b</sup>	3.43 ± 0.38 <sup>a,b</sup>	4.45 ± 0.37 <sup>b</sup>
Endrin	7.84 ± 0.94 <sup>b</sup>	12.23 ± 1.09 <sup>b</sup>	3.94 ± 0.26 <sup>b</sup>	6.85 ± 0.55 <sup>b</sup>	3.68 ± 0.45 <sup>a,b</sup>	5.29 ± 0.60 <sup>b</sup>
Naphthalene	5.34 ± 0.71 <sup>c</sup>	10.46 ± 0.88 <sup>c</sup>	3.00 ± 0.22 <sup>a</sup>	8.26 ± 1.04 <sup>c</sup>	3.03 ± 0.47 <sup>a</sup>	4.63 ± 0.48 <sup>b</sup>
Cr(VI)	6.20 ± 0.51 <sup>b,c</sup>	12.55 ± 1.22 <sup>b</sup>	3.90 ± 0.41 <sup>b</sup>	5.81 ± 0.66 <sup>b</sup>	3.74 ± 0.31 <sup>b</sup>	4.30 ± 0.61 <sup>b</sup>

DNA fragmentation was determined in brain tissues of C57BL/6NTac and p53-deficient mice 24 h after treatment with a 0, 0.01, 0.10 or 0.50 LD<sub>50</sub> dose of the four xenobiotics. Each value is the mean ± SD of four experiments.<sup>a-c</sup> Values within a column with non-identical superscripts are significantly different ( $p < 0.05$ ) from each other.

C57BL/6NTac animals (Table 27.2). Similar results were obtained in brain lipid peroxidation and DNA fragmentation (Tables 27.3 and 27.4) (Bagchi *et al.*, 2000).

Treatment of C57BL/6NTac mice with 0.50 LD<sub>50</sub> dose of TCDD, endrin, naphthalene and Cr(VI) increased cytochrome *c* reduction by 5.7-, 5.4-, 5.3- and 4.1-fold in brain tissues compared to corresponding values in untreated control animals, although under the same conditions, approximately 8.1-, 10.1-, 9.6- and 7.7-fold increases in cytochrome *c* reduction were observed in brain tissues of p53-deficient mice. In comparison, at a 0.50 LD<sub>50</sub> dose, TCDD, endrin, naphthalene and Cr(VI) induced 1.4-, 1.9-, 1.8- and 1.9-fold higher increases in cytochrome *c* reduction in brain tissue of p53-deficient animals than in C57BL/6NTac mice (Table 27.1) (Bagchi *et al.*, 2000). These results demonstrate the massive production of ROS and oxidative DNA damage by Cr(VI) and other xenobiotics. Similar results were obtained in brain lipid peroxidation and DNA fragmentation (Tables 27.3 and 27.4).

### Lead

Lead is another major environmental toxicant that causes neurological, hematological and gastrointestinal dysfunction. Prolonged exposure to lead may also cause reproductive impairment, hypertension and nephropathy. Furthermore, lead slows nerve conduction, alters calcium homeostasis, inhibits enzymes and stimulates synthesis of binding proteins (Goyer, 1996; Stohs and Bagchi, 1995). Although lead is not considered a transition metal, the catalysis of peroxidative reactions by lead may be a major contributor to the toxic effects of this metal. Various studies have shown that administration of lead to experimental animals results in the production of lipid peroxidation in the brain tissues. Administration of vitamin E prevents enhanced lipid peroxidation and toxicity of lead (Ramstoeck *et al.*, 1980), providing additional evidence for the role of oxidative stress in lead toxicity. As observed with cadmium and nickel, lead produces a compensatory increase in tissue glutathione levels. Results involving the role of ROS and oxidative stress in the toxicity of lead are consistent with the results for other heavy metals (Stohs and Bagchi, 1995).

### Iron, copper, manganese and zinc

The brain, particularly the gray matter, is rich in metal ions such as iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn), which may consist of significant concentrations within the range 0.1–0.5 mM (Hamai *et al.*, 2001). Experimental data have shown these metal ions are key factors in both the generation and defense against ROS and degenerative diseases. In neurological diseases, an abnormal reaction occurs between a protein and a redox-active metal ion promoting the formation of ROS, which can be detrimental to the nervous system and other organs (Jenner, 1996). ROS and oxidative stress significantly contribute to the pathophysiology of a broad spectrum of degenerative diseases (Cheng and Trombetta, 2004). Other studies have shown that rabbits fed a high-cholesterol diet in the presence of copper ions developed amyloid brain lesions and cognitive deficiencies (Bush *et al.*, 2003; Sparks and Schreurs, 2003).

### Oxidative stress by organophosphate and carbamate pesticides

Residential and industrial use of organophosphate and carbamate pesticides is widespread in the United States. According to the 1997 findings of the U.S. Environmental Protection Agency, over 40 organophosphate pesticides and 22 carbamate pesticides are the among 900 pesticides registered for use in the United States which pose the highest risks to animal health (Bagchi *et al.*, 2006). Organophosphates have been used extensively to control wide range of (sucking and chewing) pests of field crops, fruits and vegetables. The widespread use of the well-known organophosphate pesticide chlorpyrifos has raised major concerns about its potential to cause fetal or neonatal neurobehavioral damage, even at doses that do not evoke acute toxicity. Chlorpyrifos has been shown to inhibit replication of brain cells, to elicit alterations in neurotrophic signaling, governing cell differentiation and apoptosis, as well as to evoke oxidative stress (Bagchi *et al.*, 2006).

Recent studies indicate that toxic manifestations induced by these pesticides are associated with the



enhanced production of ROS which provides an explanation for the multiple types of toxic responses as well as a characteristic wasting syndrome. Most of these pesticides may also serve as common mediators in the activation of protein kinase C, oncogene expression, apoptosis and tumor formation. The ability to produce ROS *in vivo* with successive tissue damage was examined by brain lipid peroxidation and DNA single-strand breaks (DNA-SSB). Chemiluminescence, lactate dehydrogenase (LDH) leakage and DNA-SSB were assessed to determine the *in vitro* production of ROS. Organophosphates have also shown the mechanism involved in the induction of oxidative tissue damaging effects, including lipid peroxidation and nuclear DNA-SSB in *in vivo* models.

A comparative *in vitro* and *in vivo* effect of organophosphates such as chlorpyrifos and fenthion were assessed in cultured neuroactive PC-12 cells and in treated animals (Bagchi *et al.*, 1995a, b). Dissimilar polyhalogenated cyclic hydrocarbons, such as endrin and chlordane, and chlorinated acetamide herbicides, such as alachlor, were also compared to these organophosphates insecticides.

The ability to produce ROS *in vivo* with successive tissue damage was examined by hepatic and brain lipid peroxidation and DNA single-strand breaks (SSB). Chemiluminescence, lactate dehydrogenase (LDH) leakage and DNA-SSB were assessed to determine the *in vitro* production of ROS. These results clearly demonstrated that administration of organophosphates result in the *in vitro* and *in vivo* induction of brain lipid peroxidation, chemiluminescence response, LDH leakage and DNA-SSB, suggesting that the ROS and/or free radicals may be involved in the toxic manifestations of organophosphates and other structurally dissimilar pesticides. Furthermore, results indicate the tissue specificity of organophosphates with respect to the responses.

### *In vitro and in vivo generation of ROS and lipid peroxidation in the brain tissue by structurally dissimilar pesticides*

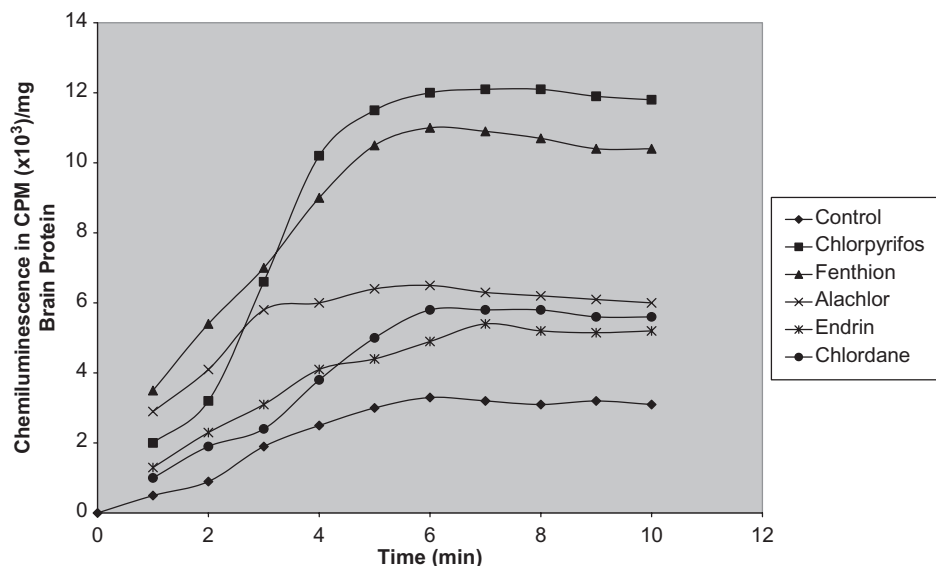
Organophosphate- and other xenobiotics-induced *in vivo* production of ROS and membrane damage in the brain tissue were assessed by brain lipid peroxidation and DNA damage. Endrin, chlordane, alachlor, chlorpyrifos and fenthion were administered orally to female Sprague-Dawley rats in two 0.25 LD<sub>50</sub> doses at 0 and 21 h and killed at 24 h. Lipid peroxidation assays were determined on the brain whole homogenates from control and treated animals according to the method of Buege and Aust (1972), based on the formation of thiobarbituric acid reactive substances (TBARS). Following treatment of the rats with chlorpyrifos, fenthion, endrin, chlordane and alachlor approximately 4.6- to 5.3-fold increases in brain lipid peroxidation were observed in brain homogenates. Similar results were obtained in cultured PC-12 cells using these pesticides. The effects of these pesticides on lipid peroxidation and DNA damage in brain homogenates are summarized in Table 27.5 (Bagchi *et al.* 1995a, b).

The effect of dichlorvos exposure on the lipid peroxidation and antioxidant defense system in different regions of the rat brain was investigated. An inhibition of acetylcholinesterase activity was used as an index of dichlorvos neurotoxicity. Dichlorvos exposure resulted in a significant decrease in glutathione peroxidase activity. The decreased levels of both reduced and oxidized glutathione as observed following dichlorvos exposure affected the GSH/GSSG ratio. The results also supported that the enzymes SOD and catalase may enhance the disposal of potentially toxic free radicals. Furthermore, the decrease in GSH levels may be a mechanism for the detoxification of dichlorvos in the brain and other organs (Julka *et al.*, 1992).

TABLE 27.5 Effects of pesticides on nuclear DNA-single strand breaks (DNA-SSB) in brain tissue and cultured PC-12 cells and lipid peroxidation (TBARS content) in Sprague-Dawley rats

Treatment	DNA-SSB (elution constant $\times 10^{-3}$ )		TBARS (nmol/mg protein)
	Brain tissue	Cultured PC-12 cells	
Control	7.9 $\pm$ 2.1 <sup>a</sup>	3.4 $\pm$ 0.4 <sup>a</sup>	5.4 $\pm$ 0.6 <sup>a</sup>
Endrin	15.0 $\pm$ 2.3 <sup>b</sup>	8.5 $\pm$ 0.9 <sup>b</sup>	12.7 $\pm$ 0.9 <sup>b</sup>
Chlordane	13.0 $\pm$ 1.0 <sup>b,c</sup>	7.6 $\pm$ 0.7 <sup>b</sup>	11.4 $\pm$ 1.1 <sup>b</sup>
Alachlor	17.0 $\pm$ 1.7 <sup>b</sup>	7.2 $\pm$ 0.6 <sup>b</sup>	19.6 $\pm$ 1.3 <sup>c</sup>
Fenthion	11.0 $\pm$ 0.8 <sup>c</sup>	8.6 $\pm$ 0.3 <sup>b</sup>	28.6 $\pm$ 2.0 <sup>d</sup>
Chlorpyrifos	11.0 $\pm$ 1.6 <sup>c</sup>	8.3 $\pm$ 0.5 <sup>b</sup>	25.0 $\pm$ 2.1 <sup>d</sup>

Female Sprague-Dawley rats received two 0.25 LD<sub>50</sub> doses of the pesticides in corn oil at 0 and 21 h and killed at 24 h. Control animals received the vehicle. Effects of 100 nM concentrations of the pesticides on DNA-single strand breaks (DNA-SSB) of cultured PC-12 cells at 24 h post-treatment were determined. DNA-SSB were determined by the alkaline elution method and are expressed as DNA elution constants. Lipid peroxidation was determined as the content of TBARS, using MDA as the standard. Each value is the mean  $\pm$  SD of at least 4–6 animals in each group. Values with non-identical superscripts are significantly different ( $p < 0.05$ ).



**FIGURE 27.1** Chemiluminescence production by brain homogenates *in vitro* following exposure to structurally diverse pesticides.

#### *Chemiluminescence response by chlorpyrifos, fenthion and other structurally diverse pesticides for generation of ROS*

The chemiluminescence assay is a non-specific test for the identification of ROS. The sustained chemiluminescence produced by brain tissues following treatment with chlorpyrifos, fenthion and other pesticides including alachlor, chlordane and endrin is presumably due to the continued production of ROS. The chemiluminescence responses produced following *in vitro* administration of these pesticides in brain tissues are shown in Figure 27.1. The chemiluminescence response produced by brain homogenates from pesticide(s)-treated rats rapidly increases, reaching a maximum between 6 and 8 minutes of incubation, while brain homogenates from control animals reach a peak chemiluminescence at 6 minutes. The chemiluminescence persisted for over 10 minutes. Increases of 2.9- and 2.4-fold were observed in the chemiluminescence responses in the brain homogenates of the animals treated with chlorpyrifos and fenthion. The greatest chemiluminescence responses in brain tissues were induced by chlorpyrifos (Bagchi *et al.*, 1995b, 2002).

#### *Lactate dehydrogenase leakage by chlorpyrifos, fenthion and other pesticides*

Organophosphates produce toxicological effects due to their ability to accumulate in the adipose tissue. Organophosphates exhibit similar abilities as inducers of hepatic drug metabolizing enzymes (Viviani *et al.*, 1978) and act as potent competitive and stereospecific inhibitors of ligand binding to specific types of brain receptors (Botham, 1990).

As an index of membrane and cellular damage, the release of lactate dehydrogenase (LDH) from cultured

PC-12 cells was measured as a function of concentration of the pesticides. Increased release of LDH into the media of cultured cells is indicative of cellular and membrane damage. Cultured PC-12 cells were incubated in the presence of 0, 50, 100 or 200 nM of chlorpyrifos, fenthion and other pesticides, and the release of LDH by the cells was measured after 24 h of incubation as an index of cytotoxicity. The amount of LDH released by these pesticides was concentration dependent. However, the differences in the release of LDH into the media were not significantly different at concentrations of 100 and 200 nM. Maximal release of LDH from cultured PC-12 cells was observed at 100 nM concentrations of the pesticides. Increases of 2.3-, 2.5-, 2.8-, 3.1- and 3.4-fold were observed in LDH leakage following incubation of the PC-12 cells with endrin, chlordane, alachlor, chlorpyrifos and fenthion, respectively (Table 27.6) (Bagchi *et al.*, 1995b, 2002).

An SDS polyacrylamide gel electrophoresis study demonstrated the overexpression of 60 and 90 kDa protein in hepatic, brain and lung tissues following treatment with structurally dissimilar pesticides (Figure 27.2). Enhanced expression of 90 kDa protein was also observed in cultured PC-12 cells following treatment with these pesticides (Figure 27.2) (Bagchi *et al.*, 1996b, 2002). Table 27.7 depicts the radioactivity ( $^{32}\text{P}$  counts/min) associated with Hsp89 $\alpha$  and Hsp89 $\beta$  mRNA expression in control and pesticide-treated cultured PC-12 cells. The cultured PC-12 cells were incubated with 50, 100 or 200 nM concentrations of the pesticides and the concentration-dependent effects of the pesticides were determined. At various concentrations of alachlor, endrin, chlorpyrifos and fenthion, the expression of Hsp89 $\alpha$  and Hsp89 $\beta$  significantly increased as compared to the control values.

TABLE 27.6 Concentration-dependent effects of pesticides on the release of LDH from cultured PC-12 cells

Treatment	Concentration of LDH in the media (U LDH/l)		
	50nM	100nM	200nM
Control	77.4 ± 9.2 <sup>a</sup>	76.9 ± 10.3 <sup>a</sup>	79.2 ± 8.6 <sup>a</sup>
Endrin	123.8 ± 16.5 <sup>b,c</sup>	178.1 ± 11.6 <sup>b</sup>	183.6 ± 20.2 <sup>b</sup>
Chlordane	100.6 ± 9.8 <sup>b</sup>	193.5 ± 20.4 <sup>b,c</sup>	212.4 ± 19.5 <sup>b,c</sup>
Alachlor	131.7 ± 11.4 <sup>c</sup>	216.7 ± 19.3 <sup>c</sup>	230.3 ± 22.6 <sup>c</sup>
Chlorpyrifos	147.1 ± 15.3 <sup>c,d</sup>	239.9 ± 20.8 <sup>c,d</sup>	241.0 ± 25.7 <sup>c,d</sup>
Fenthion	162.5 ± 13.7 <sup>d</sup>	263.2 ± 24.6 <sup>d</sup>	266.5 ± 20.4 <sup>d</sup>

PC-12 cells (25 × 10<sup>4</sup> cells/35mm Petri dish) in 2ml of RPMI 1640 were incubated for at least 3h to allow cell adherence and 50nM, 100nM, or 200nM concentrations of pesticides were added to the cultures in two equally divided portions at 0 and 24h. The incubation was continued at 37°C in an atmosphere of 5% CO<sub>2</sub> for 24h. Media were collected from the cultures and assayed for LDH activity. Data are expressed as the mean value of six experiments ± SD. Values with non-identical superscripts are significantly different (P < 0.05).

Genotoxicity of organophosphate

The detection of genotoxicity caused by organophosphate pesticides has also been determined using the single cell gel electrophoresis assay or comet assay. Chlorpyrifos and acephate were tested for their ability to induce *in vivo* genotoxic effect in leukocytes of Swiss albino mice (Rahman *et al.*, 2002). The mice were treated orally with doses ranging from 0.28 to 8.96mg/kg body weight of chlorpyrifos, or 12.25 to 392.00mg/kg body weight of acephate. A comet assay was performed on whole blood at 24, 48, 72 and 96h. A significant increase in mean comet tail length indicating DNA damage was observed at 24h post-treatment (P < 0.05) with both pesticides in comparison to cyclophosphamide (positive control) demonstrating the DNA damage was dose related. The mean comet tail length revealed a clear dose-dependent increase.

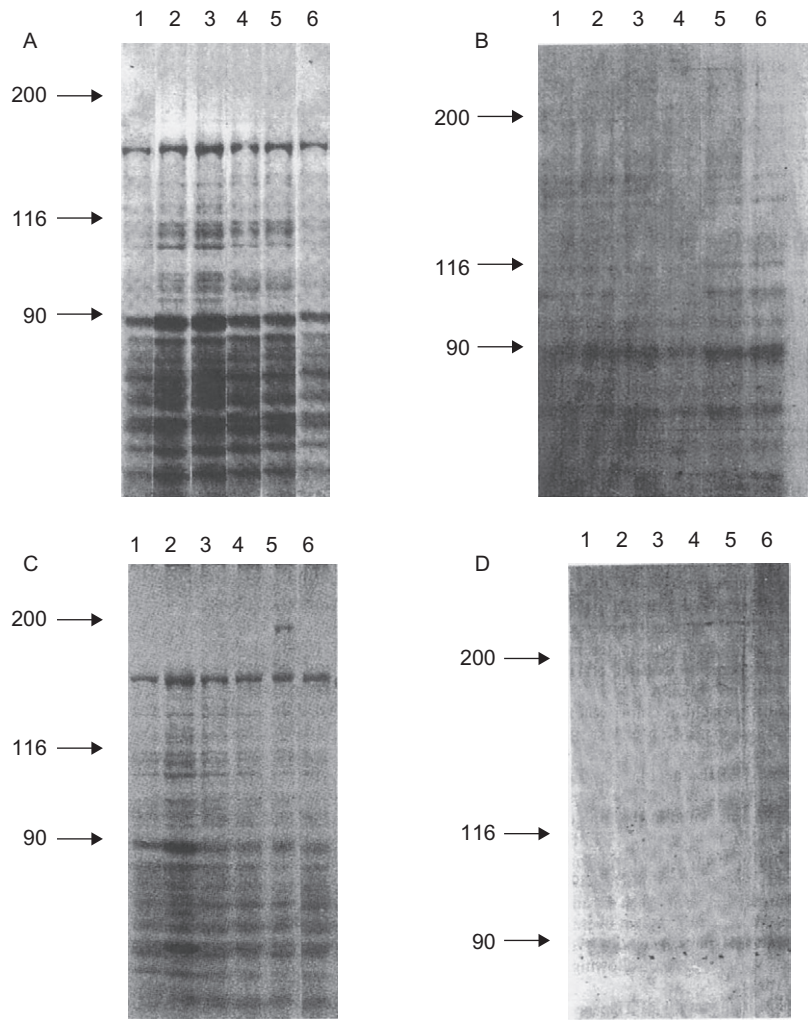


FIGURE 27.2 SDS polyacrylamide gel electrophoresis of the hepatic (a), brain (b) and lung (c) tissues from control and pesticide-treated rats, and cell lysates (d) from control and pesticide-treated cultured PC-12 cells. Lanes as follows: 1, control sample; 2, alachlor-treated sample; 3, endrin-treated sample; 4, chlordane-treated sample; 5, chlorpyrifos-treated sample; and 6, fenthion-treated sample.

TABLE 27.7 Radioactivity ( $^{32}\text{P}$  counts/min) associated with Hsp89 $\alpha$  and Hsp89 $\beta$  mRNA expression in control and pesticide-treated, cultured PC-12 cells

Treatment	Hsp89 $\alpha$			Hsp89 $\beta$		
	50nM	100nM	200nM	50nM	100nM	200nM
Control (DMSO)	637 $\pm$ 52 <sup>a</sup>	716 $\pm$ 73 <sup>a</sup>	754 $\pm$ 66 <sup>a</sup>	596 $\pm$ 34 <sup>a</sup>	634 $\pm$ 39 <sup>a</sup>	787 $\pm$ 55 <sup>a</sup>
Alachlor	2427 $\pm$ 211 <sup>b</sup>	3219 $\pm$ 255 <sup>b</sup>	3957 $\pm$ 265 <sup>b</sup>	2392 $\pm$ 181 <sup>b</sup>	3611 $\pm$ 263 <sup>b</sup>	4456 $\pm$ 328 <sup>b</sup>
Endrin	2042 $\pm$ 187 <sup>c</sup>	3315 $\pm$ 308 <sup>b</sup>	2821 $\pm$ 257 <sup>c</sup>	1808 $\pm$ 178 <sup>b</sup>	3544 $\pm$ 295 <sup>b</sup>	4030 $\pm$ 413 <sup>b</sup>
Chlordane	787 $\pm$ 64 <sup>d</sup>	893 $\pm$ 62 <sup>c</sup>	987 $\pm$ 71 <sup>d</sup>	682 $\pm$ 71 <sup>c</sup>	756 $\pm$ 84 <sup>c</sup>	941 $\pm$ 90 <sup>c</sup>
Chlorpyrifos	2287 $\pm$ 218 <sup>b,c</sup>	3626 $\pm$ 275 <sup>b</sup>	4573 $\pm$ 422 <sup>b</sup>	2938 $\pm$ 232 <sup>b,d</sup>	3885 $\pm$ 367 <sup>b,d</sup>	4744 $\pm$ 411 <sup>b</sup>
Fenthion	2642 $\pm$ 223 <sup>b</sup>	3711 $\pm$ 307 <sup>b</sup>	4388 $\pm$ 414 <sup>b</sup>	3182 $\pm$ 274 <sup>d</sup>	4312 $\pm$ 323 <sup>d</sup>	4418 $\pm$ 409 <sup>b</sup>

Cultured PC-12 ( $1 \times 10^7$ ) cells were incubated individually with either 50nM, 100nM or 200nM of alachlor, chlordane, chlorpyrifos or fenthion, and total RNA was isolated after 24h using the guanidiumisothiocyanate method. The RNAs were run on 1% agarose gels, transferred to nylon membranes and Northern blot analyses were performed by using  $^{32}\text{P}$ -labeled HSP89 $\alpha$  and HSP89 $\beta$  probes. Values are expressed as mean counts/min  $\pm$  SD of 4–6 animals. Values within each column with non-identical superscripts are significantly different ( $p < 0.05$ ).

A gradual decrease in mean tail length was noted from 48h post-treatment. By 96h of post-treatment the mean comet tail length reached control levels indicating repair of the damaged DNA.

Organophosphate-induced oxidative stress at the tubular level has been hypothesized as playing a role in the pathogenesis of acute tubular necrosis. Bidrin<sup>®</sup>, an organophosphate insecticide formulation with dicrotophos as the active ingredient, has been associated with renal tubular epithelial cell (LLC-PK1) toxicity. A study conducted by Poovala *et al.* (1999) assessed lactate dehydrogenase (LDH) release,  $\text{H}_2\text{O}_2$  levels ( $\mu\text{mol}/\text{mg}$  protein per h), and malondialdehyde formation ( $\text{nmol}/\text{mg}$  protein). Results showed LDH which significantly increased with concentration and time, after exposure of the cells to 1000, 1250, 1500, 1750 and 2000ppm of Bidrin<sup>®</sup> for 6, 12, 24 and 48h. Antioxidants 2-methylaminochroman (2-MAC) and desferrioxamine reduced cell damage induced by 1250ppm of Bidrin<sup>®</sup> over a 24h incubation. The greatest reductions in the percentage of LDH were produced by desferrioxamine 2mM and 2-MAC 2.5 $\mu\text{M}$ , both significantly lower than Bidrin<sup>®</sup> alone.  $\text{H}_2\text{O}_2$  levels were significantly elevated after exposure to 1250ppm of Bidrin<sup>®</sup>. Significantly increased malondialdehyde formation compared with control was also found in Bidrin<sup>®</sup>-exposed cells indicating enhanced lipid peroxidation. Malondialdehyde generation was significantly suppressed by 2-MAC and desferrioxamine. These results demonstrated that Bidrin<sup>®</sup> can cause direct tubular cytotoxicity, and implicate, at least in part, a role for ROS and accompanying lipid peroxidation in cytotoxicity.

#### *In vitro and in vivo protein kinase c activation by structurally diverse pesticides and chromium (VI) and cadmium (II)*

Various pesticides and transition metals induce oxidative deterioration of biological macromolecules in which

protein kinase C (PKC) may play a major regulatory role. PKC is a family of isoenzymes with distinct roles in normal and pathogenic activities within cells. PKC is involved in signaling pathways mediating the regulation of many cell processes including cell differentiation, cell survival, gene expression, secretion, cytoskeletal function, and cell–cell interactions (Lord and Pongracz, 1995). Thus PKC is involved in a cascade of events associated with cell regulation which is subject to both internal and external factors. A growing body of evidence indicates that free radicals and ROS may be involved in mediating signal transduction through interaction with PKC (Brawn *et al.*, 1995; Lander *et al.*, 1995). Studies have demonstrated that PKC is rapidly activated in cells following oxidative exposure (Brawn *et al.*, 1995). For example, the modulation of PKC activity by oxidant tumor promoters and the phorbol esters is well known (Prasad and Jones, 1992; Lord and Pongracz, 1995). The modulation of PKC by carbon tetrachloride has been shown to depend on the degree of oxidative imbalance caused by various concentrations of this haloalkane.

The comparative *in vitro* and *in vivo* abilities of structurally diverse pesticides including TCDD, endrin, chlordane, lindane, DDT, alachlor, chlorpyrifos and fenthion, and chromium (VI) and cadmium (II) to modulate protein kinase C (PKC) activity were assessed in both cultured neuroactive PC-12 cells *in vitro* and in the brain tissues of these xenobiotics-treated animals (Bagchi *et al.*, 1996a, b, 1997).

Approximately 2–3.5-fold increases in PKC activities in the brain tissues were observed as compared to the control tissues (Table 27.8). Concentration-dependent effect of these xenobiotics was observed on enhanced PKC activities (2.9- to 4.3-fold) in neuroactive pheochromocytoma PC-12 cells (Table 27.9). In these *in vitro* experiments, maximum activation of PKC was also observed primarily with 100nM concentrations of these xenobiotics. No further increases in PKC activity were observed following



**TABLE 27.8** Comparative PKC activity in female Sprague-Dawley rats following treatment with pesticides, cadmium chloride (Cd(II)) and sodium dichromate (Cr(VI)) in brain

Treatment	Protein kinase C activity/ $\mu$ g of protein/min
Control	10.7 $\pm$ 1.1 <sup>a</sup>
TCDD	25.2 $\pm$ 2.9 <sup>b</sup>
Endrin	24.4 $\pm$ 3.7 <sup>b,c</sup>
Chlordane	20.8 $\pm$ 1.7 <sup>c</sup>
Lindane	22.0 $\pm$ 2.5 <sup>c</sup>
DDT	21.1 $\pm$ 1.6 <sup>c</sup>
Alachlor	19.1 $\pm$ 1.4 <sup>c,e</sup>
Chlorpyrifos	37.4 $\pm$ 4.2 <sup>d</sup>
Fenthion	35.4 $\pm$ 2.9 <sup>d</sup>
Chromium (VI)	16.7 $\pm$ 1.9 <sup>e</sup>
Cadmium (II)	26.4 $\pm$ 3.6 <sup>b</sup>

Female Sprague-Dawley rats were treated orally with two 0.25 LD<sub>50</sub> doses at 0 and 21 h with pesticides, cadmium chloride (Cd(II)) or sodium dichromate (Cr(VI)), and killed at the 24 h time point. PKC activity was monitored using a kit from Upstate Biotechnology, NY. Values with non-identical superscripts are significantly different ( $P < 0.05$ ).

**TABLE 27.9** Comparative PKC activity in cultured neuroactive PC-12 cells following treatment with pesticides, cadmium chloride (Cd(II)) and sodium dichromate (Cr(VI))

Treatment	Protein kinase C activity/ $\mu$ g of protein/min		
	50nM	100nM	200nM
Control (DMSO)	0.78 $\pm$ 0.08 <sup>a</sup>	0.77 $\pm$ 0.10 <sup>a</sup>	0.95 $\pm$ 0.13 <sup>a</sup>
TCDD	2.28 $\pm$ 0.16 <sup>b</sup>	2.31 $\pm$ 0.26 <sup>b</sup>	2.02 $\pm$ 0.15 <sup>b</sup>
Endrin	2.10 $\pm$ 0.12 <sup>b,c</sup>	2.45 $\pm$ 0.32 <sup>b</sup>	2.28 $\pm$ 0.32 <sup>b</sup>
Chlordane	1.87 $\pm$ 0.20 <sup>c</sup>	2.41 $\pm$ 0.16 <sup>b</sup>	2.12 $\pm$ 0.14 <sup>b</sup>
Lindane	1.37 $\pm$ 0.09 <sup>d</sup>	2.09 $\pm$ 0.24 <sup>b</sup>	2.21 $\pm$ 0.18 <sup>b</sup>
DDT	1.59 $\pm$ 0.22 <sup>d</sup>	2.44 $\pm$ 0.27 <sup>b</sup>	2.45 $\pm$ 0.21 <sup>b</sup>
Chlorpyrifos	2.26 $\pm$ 0.20 <sup>b</sup>	3.31 $\pm$ 0.38 <sup>c</sup>	3.22 $\pm$ 0.40 <sup>c</sup>
Fenthion	1.88 $\pm$ 0.14 <sup>c</sup>	3.26 $\pm$ 0.43 <sup>c</sup>	3.12 $\pm$ 0.35 <sup>c</sup>
Alachlor	1.86 $\pm$ 0.24 <sup>c</sup>	2.37 $\pm$ 0.28 <sup>b</sup>	2.33 $\pm$ 0.17 <sup>b</sup>
	0.2 $\mu$ M	0.4 $\mu$ M	0.6 $\mu$ M
Chromium (VI)	1.95 $\pm$ 0.22 <sup>c</sup>	3.17 $\pm$ 0.37 <sup>c</sup>	3.21 $\pm$ 0.32 <sup>c</sup>
Cadmium (II)	1.21 $\pm$ 0.14 <sup>d</sup>	2.95 $\pm$ 0.32 <sup>c</sup>	2.88 $\pm$ 0.30 <sup>c</sup>

Cultured cells were treated individually with 50, 100 or 200 nM concentrations of pesticides, or 0.2, 0.4 or 0.6  $\mu$ M concentration of cadmium chloride (Cd(II)) or sodium dichromate (Cr(VI)) in two equally divided concentrations at 0 and 21 h and incubated at 37°C. PKC activity was monitored using a kit from Upstate Biotechnology, NY, at the 24 h time point. Values with non-identical superscripts are significantly different ( $P < 0.05$ ).

treatment with 200 nM concentrations of these xenobiotics. Thus, organophosphate pesticides as well as other xenobiotics can modulate a vital component in the cell signaling pathway (Tables 27.8 and 27.9) (Bagchi *et al.*, 1997).

#### Developmental neurotoxicity of chlorpyrifos on cultured PC-12 and Gliotypic C6 cells

Studies have demonstrated that glial-type cells have been targeted by chlorpyrifos through the same multiple mechanisms that have been demonstrated for the effects

of chlorpyrifos on brain development *in vivo* (Garcia *et al.*, 2001). Post-neurogenesis, glial development continues and given that chlorpyrifos targets events in both glial cell replication and the later stages of differentiation, the vulnerable period for developmental neurotoxicity of chlorpyrifos is likely to extend well into childhood or even early adolescence. Early *in vitro* studies have also demonstrated the methods causing neurotoxicity by chlorpyrifos, by means of assessment of cultures of immature brain tissue (Roy *et al.*, 1998; Monnet-Tschudi *et al.*, 2000) or transformed neural cell lines, such as neurotypic PC-12 cell lines. PC-12 cell lines are less receptive to neurotoxins and have shown to effectively establish cell replication as a major target because they maintain a fixed pattern of mitosis until differentiation is triggered by addition of trophic factors and deletion of serum (Crumpton *et al.*, 2000). To demonstrate the antimitotic effects of chlorpyrifos, a study in two *in vitro* models, PC-12 cells and gliotypic C6 cells, was compared (Qiao *et al.*, 2001). In the first set of experiments, PC-12 and gliotypic C6 cells were exposed to chlorpyrifos or chlorpyrifos metabolites for 1 h in the absence of serum, to obviate any potential protective effect of serum proteins (Garcia *et al.*, 2001) selecting a chlorpyrifos concentration (30  $\mu$ M) which was previously found to cause robust but submaximal inhibition of DNA synthesis *in vitro*. Equimolar concentrations of chlorpyrifos-oxon also produced significant inhibition of DNA synthesis, again with gliotypic C6 cells showing a greater effect than PC-12 cells; however, chlorpyrifos-oxon was also significantly less effective than was chlorpyrifos itself (Monnet-Tschudi *et al.*, 2000).

The effects of chlorpyrifos were also compared with other cholinesterase inhibitors; using equivalent concentrations (30  $\mu$ M) of each compound demonstrated that both diazinon and physostigmine caused significant inhibition of DNA synthesis in gliotypic C6 cells. For PC-12 cells, diazinon caused a significant decrement, though smaller than the effect of chlorpyrifos, while physostigmine was ineffective. The current results are consistent with previous concepts where it was demonstrated that chlorpyrifos exerts antimitotic actions on developing neural cells independent of cholinesterase inhibition (Pope, 1999; Slotkin, 1999). It was also observed that chlorpyrifos was more effective than chlorpyrifos-oxon, despite the fact that the latter is a far more potent cholinesterase inhibitor.

The effects of chlorpyrifos and its major metabolites in two *in vitro* models, PC-12 cells and gliotypic C6 cells, showed chlorpyrifos inhibited DNA synthesis in both cell lines but had a greater effect on gliotypic cells. Chlorpyrifos-oxon, the active metabolite that inhibits cholinesterase, also decreased DNA synthesis in PC-12 and gliotypic C6 cells with a preferential effect on the latter. However, diazinon also inhibited DNA synthesis with predilection toward gliotypic C6 cells and was

less effective than was chlorpyrifos. It was also found that the addition of sera protected the cells from the adverse effects of chlorpyrifos and that the effect could be reproduced by addition of albumin. These results indicate that chlorpyrifos and diazinon have immediate, direct effects on neural cell replication, preferentially for glial cells. The fact that the fetus and newborn possess lower concentration of serum protein, and hence at very low concentration of chlorpyrifos the neurotoxic effect occur on them, while at the same concentration these are non-toxic to adults demonstrating the protective effect of serum proteins. (Aschner, 1999; Monnet-Tschudi *et al.*, 2000; Garcia *et al.*, 2001).

Adverse effects of chlorpyrifos on glial cell replication are of critical importance in defining the sensitive period for effects on CNS development. Glial cells provide nutritional, structural and homeostatic support essential to architectural modeling of the brain (Morita *et al.*, 1999; Barone *et al.*, 2000) and because glial development continues well into the postnatal period, glial targeting implies a prolonged vulnerability, extending into childhood. Chlorpyrifos administration *in vivo* inhibits DNA synthesis and causes loss of brain cells during gliogenesis (Dam *et al.*, 1998) with maximal effects on neural functions appearing during peaks of glial development (Monnet-Tschudi *et al.*, 2000). In aggregating brain-cell cultures, chlorpyrifos affects glial markers, again unrelated to cholinesterase inhibition. The above results thus confirm conclusively that chlorpyrifos, rather than its active metabolite chlorpyrifos-oxon, is the primary agent in these effects (Bagchi *et al.*, 2006).

Taking together, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), endrin, chlordane, lindane, alachlor, naphthalene, Cr(VI) and Cd(II) are environmental as well as industrial pollutants, and extensive research has provided evidence that ROS and oxidative stress are involved in the toxicity of these xenobiotics in the brain tissue and other essential organs.

### Naphthalene (a bicyclic aromatic hydrocarbon)

Naphthalene (NAP) is widely used in various commercial and industrial applications including lavatory scent disks, soil fumigants and moth balls. Exposure to naphthalene is associated with the development of hemolytic anemia in humans and laboratory animals (Germansky and Jamall, 1988). The toxic manifestations induced by naphthalene appear to involve the conversion of naphthalene to the naphthoquinones as 1,2-naphthoquinone and 1,4-naphthoquinone (Lubek *et al.*, 1989) as well as hydroxylated products including 1-naphthol, 2-naphthol and 1,2-dihydro-1,2-dihydroxynaphthalene (Seaton and Tjeerdema, 1995). In general, quinones are believed to be

toxic by a mechanism involving redox cycling and oxidative stress. The role of ROS in the cytotoxicity of various quinoline derivatives has been reviewed extensively. Menadione (2-methyl-1,4-naphthoquinone) has been the most extensively studied model compound with respect to the toxicity of quinoline derivatives and naphthoquinones (Stohs, 1995).

### Naphthalene-induced membrane microviscosity

In a study by Vuchetich *et al.*, (1996), membrane fluidity studies were performed on mitochondrial and microsomal membranes of brain tissues by steady-state fluorescence spectroscopy. The membranes were treated with 0.5mM diphenyl-hexatriene (DPH) in tetrahydrofuran as the fluorescent probe and incubated for 2h at 37°C. The membranes were kept at 4°C for 3–5h for complete incorporation of DPH. Fluorescence polarization, a measure of membrane fluidity, was determined at 25°C with a Perkin-Elmer spectrofluorometer equipped with perpendicular and parallel polarizers, using an excitation wavelength of 365nm and an emission wavelength of 430nm. Fluorescence polarization and the apparent microviscosity were calculated as described by Shintzky and Barrenholz (1978) and Bagchi *et al.* (1992) (Table 27.10).

The ability of naphthalene to induce an oxidative stress was determined by measuring lipid peroxidation (TBARS), DNA-SSB, glutathione depletion and membrane microviscosities of the brain tissues. The effects of naphthalene (1100mg/kg) and/or vitamin E succinate administered to rats were assessed on lipid peroxidation in brain mitochondria and microsomes. Following naphthalene administration, maximum increases in lipid peroxidation in brain subcellular fractions were observed at the 12h time point. Increases in lipid peroxidation of 2.4- and 2.0-fold occurred at 12h post-treatment in the brain mitochondria and microsomes, respectively, as compared to control values (Bagchi *et al.*, 1998a).

The results indicate that naphthalene induces the production of ROS. The ROS produced may lead to enhanced lipid peroxidation, as well as other cell-damaging effects, including membrane and DNA damage and glutathione depletion, contributing to the toxic manifestations of naphthalene. The administration of vitamin E succinate significantly attenuates these effects following an acute dose of naphthalene in rats (Table 27.11).

In another study, low-dose (0.05 LD<sub>50</sub>) chronic effects of naphthalene (110mg/kg/day, po, in corn oil) were investigated for 120 consecutive days on increased lipid peroxidation and DNA fragmentation in brain tissues of female Sprague-Dawley rats. The results of this study demonstrated that chronic administration of naphthalene at low dose may induce oxidative stress leading to lipid peroxidation and DNA fragmentation in the brain and liver (Bagchi *et al.*, 1998a, b).

TABLE 27.10 NAP-induced changes of membrane microviscosity in the brain mitochondrial and microsomal membranes, and the protective ability of vitamin E succinate (VES)

Treatment	Membrane microviscosity (in Poise)			
	Mitochondria	% Control	Microsomes	% Control
<b>12h</b>				
Control	0.161 ± 0.028 <sup>a,d</sup>	–	0.183 ± 0.014 <sup>a</sup>	
VES	0.193 ± 0.023 <sup>a,c</sup>	120	0.119 ± 0.032 <sup>b</sup>	65
NAP	0.336 ± 0.060 <sup>b</sup>	209	0.320 ± 0.030 <sup>c</sup>	175
NAP + VES	0.231 ± 0.037 <sup>c,d</sup>	143	0.245 ± 0.036 <sup>d</sup>	134
<b>24h</b>				
Control	0.182 ± 0.004 <sup>a</sup>	–	0.179 ± 0.009 <sup>a</sup>	
VES	0.159 ± 0.007 <sup>d</sup>	87	0.117 ± 0.010 <sup>b</sup>	65
NAP	0.335 ± 0.013 <sup>b</sup>	184	0.295 ± 0.012 <sup>c</sup>	165
NAP + VES	0.208 ± 0.009 <sup>c</sup>	114	0.220 ± 0.005 <sup>d</sup>	123
<b>48h</b>				
Control	0.182 ± 0.005 <sup>a</sup>	–	0.160 ± 0.011 <sup>a</sup>	
VES	0.073 ± 0.290 <sup>a</sup>	95	0.121 ± 0.019 <sup>b</sup>	76
NAP	0.259 ± 0.022 <sup>d</sup>	142	0.210 ± 0.012 <sup>c</sup>	131
NAP + VES	0.211 ± 0.065 <sup>c,d</sup>	116	0.170 ± 0.035 <sup>d</sup>	106

Female Sprague-Dawley rats were individually treated with vitamin E succinate (VES) 100mg/kg for three consecutive days and 40mg/kg on days 4, 5, 6 and 7. NAP (1100mg/kg) in corn oil was administered on day 4, 2h after VES, and the animals were killed 12, 24, 48 and 72h after NAP administration. Control animals received the corresponding vehicle. Each value represents the mean ± SD of 4–6 animals. Values within each column with non-identical superscripts are significantly different ( $p < 0.05$ ).

TABLE 27.11 NAP-induced DNA single-strand breaks (DNA-SSB) and glutathione depletion in the brain nuclei and the protective ability of vitamin E succinate (VES)

Groups	DNA-SSB in brain (elution constant × 10 <sup>-3</sup> )	% Control	Glutathione depletion	% Control
<b>12h</b>				
Control	7.9 ± 0.6 <sup>a</sup>	–	3.61 ± 0.48 <sup>a</sup>	–
VES	15.0 ± 3.0 <sup>b</sup>	190	3.92 ± 0.47 <sup>a</sup>	109
NAP	19.0 ± 0.9 <sup>c</sup>	241	2.33 ± 0.41 <sup>b</sup>	65
NAP + VES	15.0 ± 3.0 <sup>b</sup>	190	2.72 ± 0.36 <sup>b,d</sup>	75
<b>24h</b>				
Control	7.0 ± 0.5 <sup>a</sup>	–	3.82 ± 0.51 <sup>a</sup>	–
VES	13.0 ± 5.0 <sup>b</sup>	186	3.54 ± 0.29 <sup>a</sup>	93
NAP	17.0 ± 8.0 <sup>b,c</sup>	243	1.96 ± 0.30 <sup>c</sup>	51
NAP + VES	15.8 ± 7.5 <sup>b,c</sup>	226	3.03 ± 0.38 <sup>d</sup>	79
<b>48h</b>				
Control	7.3 ± 0.6 <sup>a</sup>	–	3.54 ± 0.37 <sup>a</sup>	–
VES	16.3 ± 0.4 <sup>b</sup>	223	3.6 ± 0.42 <sup>a</sup>	102
NAP	16.0 ± 6.0 <sup>b,c</sup>	219	2.15 ± 0.36 <sup>b,c</sup>	61
NAP + VES	13.8 ± 0.7 <sup>b</sup>	189	3.26 ± 0.45 <sup>a,b</sup>	92
<b>72h</b>				
Control	7.5 ± 0.5 <sup>a</sup>	–	3.88 ± 0.50 <sup>a</sup>	–
VES	16.5 ± 9.0 <sup>b</sup>	220	3.72 ± 0.44 <sup>a</sup>	96
NAP	17.0 ± 9.0 <sup>b,c</sup>	227	2.42 ± 0.38 <sup>b</sup>	62
NAP + VES	16.0 ± 0.3 <sup>b</sup>	213	3.47 ± 0.42 <sup>a</sup>	89

Female Sprague-Dawley rats were individually treated with vitamin E succinate (VES) 100mg/kg for three consecutive days and 40mg/kg on days 4, 5, 6 and 7. NAP (1100mg/kg) in corn oil was administered on day 4, 2h after VES, and the animals were killed 12, 24, 48 and 72h after NAP administration. Control animals received the corresponding vehicle. Each value represents the mean ± SD of 4–6 animals. Values within each column with non-identical superscripts are significantly different ( $p < 0.05$ ).

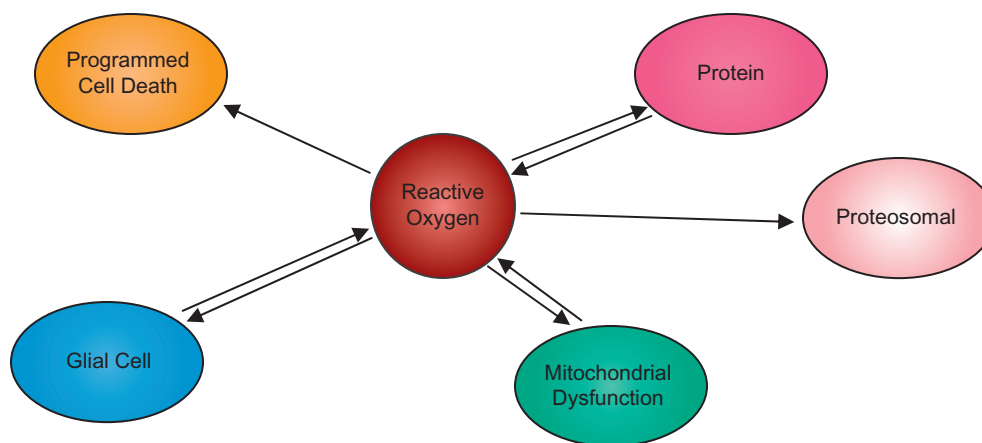


FIGURE 27.3 ROS production as a major player in the cycle of events leading to neurodegeneration.

### MYCOTOXINS, OXIDATIVE STRESS, DNA DAMAGE AND APOPTOSIS

Among all mycotoxins, aflatoxins occur with greatest frequencies contaminating animal feed. Aflatoxin B1 (AFB1) is the most toxic and is a recognized pulmonary and hepatic carcinogen. The most widely accepted mechanism of AFB1-induced carcinogenicity involves its bioactivation to AFB1-8,9-*exo*-epoxide and binding to DNA to form AFB1-N<sup>7</sup>-guanine. Another potential cause of DNA damage is AFB1-mediated excess reactive oxygen species (ROS) formation, leading to oxidation of DNA bases. Guindon *et al.* (2007) demonstrated the ability of AFB1 to cause oxidative DNA damage in lung cell types of the A/J mouse. Furthermore, the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in freshly isolated mouse lung alveolar macrophages, alveolar type II cells and nonciliated bronchial epithelial (Clara) cells was assessed by high-performance liquid chromatography with electrochemical detection. These results support the possibility that oxidative DNA damage in mouse lung cells contributes to AFB1 carcinogenicity.

Both AFB1 and ochratoxin A (OTA) induce oxidative stress and play an important role in the toxicity and cellular injury. The protective effect of two catechins, namely, epigallocatechin gallate and epicatechin gallate, against ochratoxin A-induced cytotoxicity was investigated in a pig kidney cell line (LLC-PK1) (Costa *et al.*, 2007). The ability of catechins to reduce ochratoxin A-induced oxidative stress and DNA damage was investigated. The free-radical scavenging capacity of both catechins was tested with the Briggs-Rauscher oscillating method and the TEAC assay. Significant reduction of ochratoxin A-induced oxidative stress and DNA fragmentation was found in cultured LLC-PK1 cells pretreated with catechins.

Elimination of AFB1 contamination in agricultural products has been difficult; the use of natural or synthetic free radical scavengers could be a potential chemopreventive strategy. Boric acid is the major component of industry and its antioxidant role has recently been reported. Turkez and Geyikoglu (2010) assessed the protective ability of boric acid following exposure to AFB1 in human whole blood cultures. The biochemical characterizations of glutathione and some enzymes have been carried out in erythrocytes. Alterations in malondialdehyde (MDA) level were determined as an index of oxidative stress. The sister-chromatid exchange and micronucleus tests were performed to assess DNA damages in lymphocytes. AFB1 treatment significantly reduced the activities of antioxidants by increasing MDA level (30.53 and 51.43%) of blood, whereas the boric acid led to an increased resistance of DNA to oxidative damage induced by AFB1 in comparison with control values ( $P < 0.05$ ). In conclusion, the support of boric acid was especially useful in AFB1-toxicated blood. Thus, the risk on tissue targeting of AFB1 could be reduced ensuring early recovery from its toxicity.

### CONCLUSIONS

Oxidative stress-related degenerative diseases constitute one of the major challenges of modern medicine and health professionals. Although these diseases are relatively common and often highly debilitating, the mechanisms responsible for their clinical pathologies are poorly understood, and there are currently no effective preventive or therapeutic strategies. Recent studies demonstrated that oxidative stress and oxidative DNA damage may be involved in the cytotoxicity of chlorpyrifos,



fenthion and other pesticides, as well as heavy metals. Reactive oxygen species (ROS) may serve as common mediators in programmed cell death (apoptosis) or non-programmed cell death (necrosis) in response to many toxicants and pathological conditions. Figure 27.3 exhibits a schematic diagram of the mechanistic pathways involved in the disease pathophysiology. Finally, much experimental evidence demonstrates that structurally diverse and dissimilar environmental pollutants including pesticides, heavy metals and mycotoxins have adverse effects on immune functions and various organs in the body. These cascades of events lead to altered gene expression and down-regulation of antioxidant defense system and cause chemical toxicity. Lowering the burden of protein aggregation, oxidative and nitrosative stress, mitochondrial injury, inflammatory response and heavy metal toxicity in the body to block chemical toxicity may prove beneficial in the treatment of several degenerative diseases due to oxidative stress and mycotoxin-induced toxicity. The economic impact of mycotoxin and other pesticide-related chemical toxicity include loss of animal and human life, increased health care and veterinary care costs, reduced livestock production, disposal of contaminated foods and feeds, and investment in research and applications to reduce severity of the above problems. Although efforts have continued internationally to set guidelines to control mycotoxins, pesticides, herbicides and metal toxicity, practical measures have not been adequately implemented.

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# Toxicity of over-the-counter drugs

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## INTRODUCTION

The topic of over-the-counter (OTC) drugs is complicated, encompassing a large number of products, many containing multiple active ingredients. Products are available for oral, topical, intraocular, intranasal and intrarectal administration, though most veterinary exposures are through ingestion. These products are readily available in many homes. Toxicosis may result when animals are medicated by well-intentioned animal owners or even veterinarians, or exposure may be accidental, in which case the amount of medication ingested may not be known. Response to a given drug may be species specific or different between individuals of the same species. Idiosyncratic reactions to drugs must also be considered, though reports in domestic animals are rare (Papich, 1990; Brumbaugh, 2001).

Approximately a quarter of the calls to human poison control centers in 1990 dealt with OTC drugs (Murphy, 1994; Villar *et al.*, 1998). Similarly, nearly a quarter of the calls to the American Society for the Prevention of Cruelty to Animals' Animal Poison Control Center (ASPCA-NAPCC) in 2010 were for human medications, most of them OTC (<http://www.asPCA.org/pet-care/poison-control/top-10-pet-poisons-of-the-year.aspx>, accessed February 26, 2011). It is estimated that there are over 300,000 OTC drug formulations available with approximately 700 active ingredients in various combinations (Papich, 1990). Only a small fraction can be addressed in this chapter.

Important classes of drugs that will be addressed include analgesics, cold, flu and allergy medications, and drugs used to treat gastrointestinal symptoms. Nutritional supplements, for the most part, will not

be addressed here, though a few herbal preparations (ma huang and guarana) are discussed. Toxicologically important minerals such as iron and important vitamins such as vitamin D are addressed in other chapters. Stimulants and diet pills often contain methylxanthines such as caffeine, which are also addressed in appropriate chapters, or sympathomimetic amines, which are discussed later with decongestants.

Though this chapter deals mostly with drugs intended for *per os* (PO) dosing, a few other types of formulations are discussed as well.

## SUSPECTED OTC DRUG REACTIONS

Obtaining a thorough history is of great importance when dealing with suspect OTC drug-related problems (Talcott, 2006). Animal owners may not volunteer critical information. Well-intentioned pet owners sometimes administer over-the-counter drugs to treat perceived symptoms in their pets (Papich, 1990; Jones *et al.*, 1992; Villar *et al.*, 1998; Roder, 2004a; Sellon, 2006). Veterinarians may share the blame for inappropriate dosing. Chronic analgesic administration to treat orthopedic problems is a particular hazard affecting all species of animals that present to the veterinary practice.

Accidental ingestions occur when drugs are improperly stored. Careful assessment of the history can be challenging but may be critical since unexpected circumstances can arise. For example, severe clinical signs prompted euthanasia in a kitten but it was later discovered that the kitten had been allowed to play with an empty acetaminophen container (Allen, 2003).

If an adverse reaction to an OTC drug is suspected, administration of the drug should be immediately discontinued. The owner should be instructed to bring the drug container to the veterinarian as a source of information on the active ingredients, indications for use and manufacturer identification, telephone number or address. Many manufacturers have information on treatment and prognosis and, in the United States, will use the information you provide for adverse events reporting to the Food and Drug Administration (FDA), and thus should be contacted. More information on adverse event reporting can be found at the FDA website: <http://www.fda.gov/cvm/adetoc.htm>. Any material remaining in the container can be analyzed to verify that the contents are as expected (Brumbaugh, 2001). Serum, urine and gastric lavage (liver, kidney and GI content for postmortem) should also be saved for analysis if it is unclear what the animal has ingested. Early gastrointestinal decontamination is sometimes helpful in the asymptomatic animal and may involve emetics or gastric lavage, and instillation of activated charcoal and cathartics. If drugs are used topically, removal with a mild detergent bath is usually beneficial. Careful monitoring and maintenance of body temperature is required after bathing, and bath towels may be warmed by tumbling in a heated clothes drier to help prevent hypothermia.

## ANALGESICS

According to Jones *et al.* (1992), about 5% of dog- and cat-related calls to one poison control center were in response to analgesic ingestion, and nearly 80% involved dogs. Commonly used OTC analgesics include acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, naproxen and ketoprofen. Aspirin shares many properties with other NSAIDs, but will be addressed separately due to some of its unique features.

### Acetaminophen

Acetaminophen is an analgesic and antipyretic, though it does not have the anti-inflammatory properties of NSAIDs (Hjelle and Grauer, 1986). Acetaminophen is a metabolite of the historic drug phenacetin. Acetaminophen is found in a variety of over-the-counter pain relievers and is often combined with other drugs in cold, flu and allergy medications (Roder, 2004a). Common brand names in the U.S. include Tylenol and Anacin-3. Acetaminophen is sold under the name Paracetamol in Australia and Great Britain.

Acetaminophen toxicosis is most commonly reported in cats (Rumbeiha *et al.*, 1995). Clinical acetaminophen toxicosis is usually associated with a single exposure, though adverse effects as a result of multiple dosing have been reported (Hjelle and Grauer, 1986; Villar *et al.*, 1998). The most common clinical situation results from the owner medicating their animal without the benefit of a veterinarian's advice and supervision (Hjelle and Grauer, 1986; Aronson and Drobatz, 1996). One report documents severe poisoning in a kitten that had played with an empty acetaminophen container (Allen, 2003). Although the hepatotoxic effects of acetaminophen have been described in many species, the hematotoxic effects are specific to cats and dogs (McConkey *et al.*, 2009).

### Toxicity

Individual differences in sensitivity to acetaminophen are reported within species (Webb *et al.*, 2003), but the use of acetaminophen is always contraindicated in cats due to their extreme sensitivity (Jones *et al.*, 1992; Roder, 2004a; Villar *et al.*, 1998; Wallace *et al.*, 2002). Clinical signs of acetaminophen toxicosis, up to and including death, have been reported in cats at doses of 10 mg/kg (Aronson and Drobatz, 1996). However, most poisonings are associated with doses of 50 mg/kg and greater (Murphy, 1994; Allen, 2003; MacNaughton, 2003; Roder, 2004a; Sellon, 2006). Villar *et al.* (1998) reported 50% methemoglobinemia within 4 hours in cats dosed with 120 to 140 mg/kg acetaminophen, and one of four cats dosed with 143 mg/kg died. One regular acetaminophen tablet contains 352 mg of the active ingredient, and an extra-strength tablet contains 500 mg.

The recommended dose for acetaminophen in dogs is 15 mg/kg PO every 8 hours or 10 mg/kg PO every 12 hours (Plumb, 2005). Toxicosis has been reported at a dose of 46 mg/kg (Sellon, 2006), though doses of 100 mg/kg or greater are more likely to be associated with clinical signs (Jones *et al.*, 1992; Boothe, 2001; Roder, 2004a). Most dogs dosed with less than 460 mg/kg will recover (Villar *et al.*, 1998). Doses over 460 mg/kg are associated with methemoglobinemia in dogs and deaths have occurred (Schlesinger, 1995; Villar *et al.*, 1998; Wallace *et al.*, 2002). A dose of 900 mg/kg caused "fulminant liver failure" in a dog (Boothe, 2001). Doses greater than 1 g/kg (1000 mg/kg) are reported to cause unconsciousness and cyanosis within hours and death within 12 hours (Villar *et al.*, 1998).

### Toxicokinetics

After ingestion, acetaminophen is rapidly absorbed in the stomach and small intestine (Schlesinger, 1995; Wallace *et al.*, 2002). Peak plasma concentrations occur 4 hours after ingestion in cats (Rumbeiha *et al.*, 1995). Circulating acetaminophen is minimally bound to plasma



protein and is widely distributed. The therapeutic plasma concentration of acetaminophen in a dog is near 30 µg/mL, and toxicosis is associated with levels of 300 µg/mL and greater.

Metabolism of acetaminophen occurs primarily in the liver. There are three major pathways: direct glucuronide conjugation, direct sulfate conjugation and oxidation mediated by cytochrome P450 enzymes (Hjelle and Grauer, 1986; Wallace *et al.*, 2002). Acetaminophen has an available hydroxyl group. Immediate phase II conjugation is the primary route of metabolism in most species and involves glucuronide and sulfate (Dahm and Jones, 1996). Due to limitations of cat physiology, they have only about one-tenth the acetaminophen biotransformation ability of dogs (Hjelle and Grauer, 1986; Sellon, 2006). Glucuronide conjugation is the fate of 50 to 60% of a dose of acetaminophen given to either a human or dog (Aronson and Drobatz, 1996). This pathway is deficient in cats due to decreased microsomal UDP-glucuronosyltransferase enzyme activity (Wallace *et al.*, 2002).

The amount of acetaminophen that is conjugated to glucuronide in cats is dependent on dose, but is always a relatively small proportion. After an oral dose of 20, 60 and 120 mg/kg acetaminophen, only 1, 5 and 16% undergoes glucuronide conjugation, respectively (Hjelle and Grauer, 1986). Sulfate conjugation is less important than glucuronide conjugation in the disposition of acetaminophen in most species (Aronson and Drobatz, 1996; Boothe, 2001; Allen, 2003). Dogs metabolize about 10 to 20% of a given dose of acetaminophen via sulfate conjugation, but this is the most important, although still limited, pathway in cats. Again, use of this pathway is dependent on the dose of acetaminophen. After cats were dosed with 20 mg/kg, 92% of the acetaminophen underwent sulfate conjugation, but after a dose of 60 mg/kg acetaminophen, 78% was sulfated, and if the dose was 120 mg/kg, only 57% was sulfated. The sulfate conjugation pathway can be saturated due to the limited availability of inorganic sulfates (Hjelle and Grauer, 1986).

Metabolism of acetaminophen by phase I processes is relatively minimal in most species, but is very important to the mechanism of toxicity of this drug, as will be described in the following section. Cytochrome P450 oxidation increases as phase II pathways become saturated (MacNaughton, 2003; Sellon, 2006). Approximately 5% of a dose of acetaminophen undergoes oxidation by cytochrome P450s in the dog. When cats are dosed with 20 mg/kg acetaminophen, 5% undergoes oxidation, but this number increased to 10% at doses of 60 to 120 mg/kg (Hjelle and Grauer, 1986). The product of the oxidation pathway is N-acetyl benzoquinoneimine (NAPQI). NAPQI is conjugated to reduced glutathione (GSH), forming an inactive product. A second byproduct of acetaminophen metabolism, via phase I deacetylation, is para-aminophenol (PAP). PAP is rapidly conjugated to GSH or acetate

in laboratory rodents, but cats have reduced capacity for N-acetylation and dogs lack the hepatic N-acetyltransferase enzyme (McConkey *et al.*, 2009).

The byproducts of acetaminophen metabolism are excreted predominantly through the urine (MacNaughton, 2003; Sellon, 2006; Sturgill and Lambert, 1997). Less than 5% of a dose is excreted as the parent compound in humans (Wallace *et al.*, 2002). Some conjugates are eliminated in the bile (Sturgill and Lambert, 1997; Maddrey, 2005). Rate of elimination is dependent on the species and the dose. The elimination half-life of a 100 to 200 mg/kg dose of acetaminophen is 72 minutes in dogs and increases to 210 minutes when the dose is increased to 500 mg/kg. The elimination half-life in cats for a 20 mg/kg dose is 36 minutes, but the half-life for a 60 mg/kg dose is 144 minutes, and that for a 120 mg/kg dose is 288 minutes (Hjelle and Grauer, 1986). The half-life is longer in male cats compared to females (Rumbeiha *et al.*, 1995).

### Mechanism of action

Unlike NSAIDs, the therapeutic effects of acetaminophen are independent of cyclooxygenase (COX) based on its interference with endoperoxidase (Boothe, 2001). The main target organ of acetaminophen toxicity is liver in most species. Many of the toxic effects of acetaminophen are due to the formation of the metabolite NAPQI. Large doses of acetaminophen overwhelm the sulfide and glucuronide conjugation pathways and lead to increased formation of the active metabolite (Hjelle and Grauer, 1986; Dahm and Jones, 1996; Sturgill and Lambert, 1997; MacNaughton, 2003; Roder, 2004a). NAPQI is usually conjugated with GSH, as noted above. GSH stores become depleted 16 to 24 hours after exposure to acetaminophen due to oxidation and decreased production of GSH. Para-aminophenol (PAP), another reactive metabolite, has recently been implicated in the hematotoxic effect seen in cats and dogs (McConkey *et al.*, 2009).

Unlike most species, erythrocyte injury is the predominant adverse effect associated with acetaminophen ingestion in cats. It is also observed in dogs at high doses, although effects on liver predominate in dog toxicosis at lower doses. Oxidative injury to erythrocytes takes the form of methemoglobin production or Heinz body production (Rumbeiha *et al.*, 1995; Webb *et al.*, 2003; McConkey *et al.*, 2009). GSH becomes depleted in erythrocytes and hemoglobin is oxidized to methemoglobin, which cannot carry oxygen (Hjelle and Grauer, 1986; Aronson and Drobatz, 1996). Animals with 30% of their hemoglobin converted to methemoglobin show clinical signs and cyanosis (Rumbeiha *et al.*, 1995). Methemoglobinemia is reversible (Schlesinger, 1995). Methemoglobin reductase is the enzyme responsible for

converting methemoglobin to hemoglobin. Cats have less methemoglobin reductase activity than other domestic species (MacNaughton, 2003). Acetaminophen can produce methemoglobinemia in dogs as well as cats, but this change is not seen in other species (Hjelle and Grauer, 1986; McConkey *et al.*, 2009).

Heinz body formation is an irreversible change caused by the precipitation of hemoglobin (Schlesinger, 1995; Aronson and Drobatz, 1996). Heinz bodies increase red cell fragility and decrease survival time of erythrocytes, and thus may cause hemolysis and anemia. Until recently, it was believed that NAPQI binds to the sulfhydryl groups and oxidize hemoglobin (Allen, 2003). However, erythrocytes lack the enzymes to metabolize acetaminophen to NAPQI and circulating NAPQI is unlikely to be bioavailable and has only limited ability to oxidize hemoglobin. Another reactive metabolite, PAP, was found to oxidize hemoglobin *in vitro* and thus has recently been proposed as the cause of methemoglobinemia in cats and dogs (McConkey *et al.*, 2009). There are eight sulfhydryl groups on feline hemoglobin, but only four on the hemoglobin of other domestic species and two on the human molecule, making cat erythrocytes much more prone to oxidative injury (Hjelle and Grauer, 1986; Rumbeiha *et al.*, 1995; Aronson and Drobatz, 1996; Allen, 2003; Sellon, 2006).

Compared to the hematotoxic effects that predominate in cats, hepatotoxic effects predominate in dogs, mice, rats and humans (Wallace *et al.*, 2002; Sellon, 2006). Oxidative damage to hepatocytes leads to zone 3 (centrilobular) hepatocyte degeneration and necrosis (Hjelle and Grauer, 1986; Dahm and Jones, 1996; Treinen-Moslen, 2001; Wallace *et al.*, 2002). NAPQI acts as an electrophile, causing tissue damage through formation of covalent adducts with biological macromolecules (Savides and Oehme, 1985; Hjelle and Grauer, 1986; Jones *et al.*, 1993; Dahm and Jones, 1996; Sturgill and Lambert, 1997; Villar *et al.*, 1998; Zimmerman, 1999; Treinen-Moslen, 2001). Mitochondria could be the primary target for NAPQI, which is believed to alter mitochondrial functional integrity and bind to adenine nucleotides. NAPQI is also believed to bind to membrane proteins that regulate calcium homeostasis, thus increasing intracellular calcium concentrations. Cytoskeletal damage/activation of endonucleases and DNA fragmentation are proposed in the mechanism of cell death. Production of superoxide anions causing peroxidative injury occur during phase I metabolism of acetaminophen, causing oxidative stress in the cell, especially once glutathione has been depleted. Endothelial damage is the likely cause of clinical signs such as edema of the face and extremities and hemorrhage.

Factors that enhance the toxic effects of acetaminophen include glutathione depletion due to fasting (Treinen-Moslen, 2001) and induction of P4502E1, as occurs

with barbiturate exposure (Sturgill and Lambert, 1997). Cimetidine inhibits cytochrome P450s and has been used therapeutically for acetaminophen toxicosis. Young animals are sometimes less sensitive to acetaminophen toxicosis than mature animals. This is the case in children and neonatal mice, and is believed to be due to the immaturity of the mixed-function oxidase system and more rapid glutathione synthesis in these youngsters (MacNaughton, 2003).

### Clinical signs

Clinical signs of acetaminophen toxicosis are attributable to its toxic effects on erythrocytes and hepatocytes. Clinical methemoglobinemia is the most common problem in cats and can also occur in dogs. Centrilobular hepatic necrosis is more common in dogs and also occurs in other species, including cats, humans, rats and hamsters (Hjelle and Grauer, 1986).

Clinical signs in cats usually occur within a couple of hours of exposure. Anorexia and vomiting are reported in 35% of cats presenting for acetaminophen exposure, and hypersalivation is reported in 24% (Aronson and Drobatz, 1996), and commonly occurs within 2 hours of exposure (Savides and Oehme, 1985). Diarrhea occurred in 18% of cats. Mental depression is reported in 76% and usually takes place within 3 hours.

Methemoglobinemia occurs within the 4 hours of acetaminophen ingestion and is dose dependent. Cats given a dose of 60 mg/kg acetaminophen had 21.7% of their hemoglobin converted to methemoglobin, and the methemoglobin concentration in cats dosed with 120 mg/kg acetaminophen was 45.5% (Hjelle and Grauer, 1986). Clinically evident cyanosis occurs at about 30% methemoglobinemia. Fifty-nine percent of the cats in the Aronson and Drobatz (1996) study had pale or dark mucous membranes and respiratory distress, and blood was brown in 12%. Affected cats are weak and depressed (Hjelle and Grauer, 1986; Jones *et al.*, 1992; Allen, 2003) and 12% presented comatose and 18% tachycardic (Aronson and Drobatz, 1996). Hemolysis, anemia, icterus and pigmenturia have been described and are seen within 48 hours of exposure. In addition to characteristic methemoglobinemia, edema of the face and forelimbs or front paws is commonly described in affected cats. Death in cats is usually due to methemoglobinemia, but fatal liver failure can also occur. Hepatic necrosis is most commonly associated with high-dose exposures, particularly in male cats.

Dogs often vomit soon after ingesting a high dose of acetaminophen, which can serve a protective function (Schlesinger, 1995). Severe clinical signs in dogs are usually attributable to hepatic necrosis (Hjelle and Grauer, 1986; Schlesinger, 1995; Sellon, 2006). Signs commence within 36 hours of ingestion and include nausea

and vomiting, anorexia, abdominal pain and depression. Tachycardia and tachypnea have been reported (Roder, 2004a; Sellon, 2006). Mild cases usually recover in another two to three days, but severe cases progress to icterus and death, usually within four days (Murphy, 1994; Sellon, 2006).

Methemoglobinemia also occurs in dogs after ingesting high doses of acetaminophen, and is more likely to cause death than is liver failure. Methemoglobinemia usually occurs within 12 hours of ingestion, but some dogs present after 48 hours. Doses of 200 mg/kg acetaminophen PO converted 18.8% of hemoglobin to methemoglobin in dogs, and 500 mg/kg produced 51.5% methemoglobinemia (Hjelle and Grauer, 1986). Signs reported included cyanosis, brown blood, lethargy and recumbence (Schlesinger, 1995; Wallace *et al.*, 2002; MacNaughton, 2003). Hemolysis, anemia, icterus and shock have been described. Several authors report pigmenturia. Occasionally, signs of methemoglobinemia occur in acetaminophen-intoxicated dogs in the absence of clinically evident liver damage. Facial edema, edema of conjunctiva and nictitating membrane, and edema of the forelimbs or paws are observed in dogs.

### Clinical chemistry

Methemoglobinemia and hemolysis are noted, especially in cats. Whole blood exposed to air appears brown. Heinz bodies in cats and dogs are evident on blood smears stained with new methylene blue. Heinz bodies occur within three days of dosing (Webb *et al.*, 2003) and were evident in 12% of accidentally exposed cats (Aronson and Drobatz, 1996). Anemia occurred in 75% of cats (Aronson and Drobatz, 1996) and is reported in dogs (Schlesinger, 1995; Wallace *et al.*, 2002; MacNaughton, 2003). Hyperbilirubinemia occurred within 48 h of acetaminophen ingestion in cats and has been reported in dogs with hemolysis (Schlesinger, 1995; Sellon, 2006). Regeneration was apparent in one dog 11 days after acetaminophen ingestion.

Hypoglycemia, mild hyperbilirubinemia, elevated ALT, ALP, BUN, creatinine, and CK were the changes reported in a dog that had ingested phenylpropanolamine (Rumbeiha *et al.*, 1995; Webb *et al.*, 2003; Sellon, 2006). This change was reported by Aronson and Drobatz (1996) in 35% of exposed cats. Increased ALT in dogs is a direct result of toxic hepatic injury, though hypoxic injury may contribute, and occurs within 24 hours of acetaminophen ingestion. Increased aspartate transaminase (AST) and alkaline phosphatase (ALP) activities are reported. Evidence of severe liver damage includes elevated prothrombin time (PT), partial thromboplastin time (PTT) and progressively decreased serum cholesterol albumin concentrations (Sellon, 2006). Forty-one percent of cats presenting for acetaminophen

toxicosis had hypocholesterolemia and 12% had hypoalbuminemia (Aronson and Drobatz, 1996). Hemoglobinuria and hematuria have been observed in dogs and cats with acetaminophen toxicosis.

### Diagnosis and management

Diagnosis of acetaminophen poisoning is usually based on a history of clinical exposure and appropriate clinical signs. Plasma, serum and urine can be tested for acetaminophen at human hospitals and some veterinary laboratories to confirm the diagnosis. However, test results are usually not available for hours or days, and acetaminophen toxicosis presents as an emergency, thus treatment should be initiated immediately.

If the animal presents within 6 hours of ingestion, decontamination measures can be instituted to prevent further absorption. Emetics can be used in the alert animal within a couple of hours of ingestion. However, if large doses were ingested, gastric lavage of the anesthetized, intubated animal is more likely to be appropriate. Activated charcoal and a cathartic such as sorbitol are given to prevent further absorption.

Antidotal therapy must be initiated as soon as possible in animals suspected of ingesting a toxic dose of acetaminophen. Antidotal therapy involves use of sulfate sources to bind the active metabolites and enhance glutathione production. Several sulfur donors are available and include N-acetylcysteine, S-adenosyl methionine (SAME) and sodium sulfate. *N-acetylcysteine therapy is the most accepted treatment for acetaminophen toxicosis.* Additionally, antioxidants can be used to reduce methemoglobin.

N-acetylcysteine is a source of sulfhydryl groups to be used for phase II sulfate conjugation or for glutathione production (Hjelle and Grauer, 1986; Savides and Oehme, 1985; Villar *et al.*, 1998). N-acetylcysteine is hydrolyzed to L-cysteine by deacetylase enzymes and is oxidized in the liver to inorganic sulfate. Use of N-acetylcysteine promotes sulfate conjugation of acetaminophen metabolites in cats. Rumbeiha *et al.* (1995) reported a 50% decrease in the plasma half-life of acetaminophen in cats treated with N-acetylcysteine and Savides and Oehme (1985) reported an increase in the total fraction excreted as a sulfate conjugate. Cats treated with N-acetylcysteine have a more rapid recovery of blood GSH concentrations than untreated cats. N-acetylcysteine decreased the half-life of methemoglobin in the blood of cats from more than 10 hours to 5 hours and prevented anemia.

N-acetylcysteine therapy should be initiated to any suspected case of acetaminophen toxicosis, even if treatment is delayed or exposure is unconfirmed. This treatment is most effective within 8 hours of exposure. However, treatment within 24 hours decreases mortality



though liver damage often occurs. N-acetylcysteine is sold in 10 and 20% solutions. Twenty percent solutions are diluted 1:1 in normal saline or 5 to 10% dextrose solution (MacNaughton, 2003; Plumb, 2005; Sellon, 2006). An initial dose of 140mg/kg is given intravenously (IV) slowly. Alternately, the dose can be given orally if no vomiting is evident and activated charcoal has not been given recently. Doses of 70mg/kg N-acetylcysteine should be given IV or PO every 6 hours thereafter for 48 hours.

SAMe is another possible sulfate source used to treat acetaminophen toxicosis in dogs and cats. Decreased hemolysis and overall improvement were reported in treated dogs (Wallace *et al.*, 2002). Prevention of methemoglobin production in cats by SAMe has been inconsistent. The number of Heinz bodies was lower and the packed cell volume (PCV) remained higher in treated versus untreated experimental cats (Webb *et al.*, 2003; Sellon, 2006). Dogs are given 40mg/kg PO as their first dose and 20mg/kg daily for seven to nine days. Protocols recommended for cats include 180mg/kg SAMe PO every 12 hours for three days, and 90mg/kg SAMe PO every 12 hours for 14 days.

There are reports of sodium sulfate as a treatment for acetaminophen toxicosis. This protocol decreased the plasma half-life of acetaminophen in cats and increased the amount excreted as a sulfate conjugate, similar to N-acetylcysteine (Savides and Oehme, 1985). Severity and duration of clinical signs were decreased in treated animals, methemoglobin concentrations were decreased, and glutathione concentrations rebounded faster than in untreated cats (Villar *et al.*, 1998). Sodium sulfate was given as a 1.6% solution at a dose of 50mg/kg IV every 4 hours for six treatments.

Ascorbic acid and methylene blue are commonly used to reduce methemoglobin to hemoglobin. Ascorbic acid causes nonenzymatic reduction of methemoglobin, but is slow acting. Ascorbic acid is given at a dose of 30mg/kg PO every 6 hours for six or seven treatments. Methylene blue has a rapid onset, but can induce hemolytic anemia in cats, making it a risky choice. Still, methylene blue has been used successfully for short-term management of feline methemoglobinemia. Use of methylene blue concurrently with N-acetylcysteine in cats should be avoided.

Supportive and symptomatic therapy for acetaminophen toxicosis includes administration of oxygen to animals with methemoglobinemia. Transfusion or hemoglobin replacement is needed in cases of severe anemia (Murphy, 1994; Sellon, 2006). Fluid therapy is aimed at improving hydration, electrolyte balance and pH.

Cimetidine has been recommended for use in dogs and cats to inhibit acetaminophen metabolism through inhibition of cytochrome P450 enzymes. However, the doses required for enzyme inhibition are higher than doses used routinely (Sellon, 2006).

The time that elapses between exposure and treatment seems to be as, if not more, important in estimating the prognosis for survival. Most cats that survived were treated within 14 hours of exposure, though one was not treated for 24 hours. Most of the cats that were treated 17 or more hours post-exposure died. There were no differences in exposure-dose between the group of cats that died and those that survived. Cats with underlying disease may have a worse outcome than otherwise healthy cats. The survival rate in dogs is decreased if they are not treated within 72 hours (Sellon, 2006). Animals that were treated and survived usually recovered within 48 hours and were hospitalized for three days (Aronson and Drobatz, 1996).

### *Postmortem findings*

Icterus is a common finding secondary to acute hemolysis or chronic cholestasis. Centrilobular necrosis occurs commonly in dogs and has been described in cats, though necrosis is likely to be more diffuse in cats. Bile duct proliferation, vacuolar hepatocyte degeneration and mononuclear cholangitis are reported in dogs with chronic liver injury. Subcutaneous edema extends from the head along the fascial planes of the neck and thorax in some affected cats and dogs and can affect the conjunctiva (Allen, 2003).

## **Nonsteroidal anti-inflammatory drugs (NSAIDs)**

NSAIDs are defined as "compounds that are not steroidal and that suppress inflammation" (Boothe, 2001). These drugs have antithrombotic actions when taken at low doses, relieve minor pain and pyrexia at moderate doses and have anti-inflammatory effects at higher doses (Rubin and Papich, 1990; Boothe, 2001). NSAIDs are commonly used to treat orthopedic problems in dogs (Wallace *et al.*, 1990). About 8% of all human and veterinary related calls to the Illinois Poison Control Center were reported to involve NSAIDs, most commonly aspirin, ibuprofen, naproxen, piroxicam, indomethacin and phenylbutazone. Out of those calls, 70% of the nonhuman animal calls were for dogs and 25% for cats (Kore, 1990). NSAID toxicosis can be caused by a single large dose or multiple smaller doses (Albretsen, 2002).

There are more than 30 commercially available NSAIDs (Mazué *et al.*, 1982). They are classified based on their structure into the carboxylic acid group, which includes salicylic acid derivatives, acetic acid derivatives, fenamates or anthranilic acid derivatives and propionic acid derivatives, as well as the enolic acid groups. An incomplete list of NSAIDs is provided in Table 28.1.



TABLE 28.1 An incomplete list of nonsteroidal anti-inflammatory drugs

Carboxylic acid group	Enolic group
<b>Salicylic acid derivatives</b>	<b>Pyrazolone derivatives</b>
Aspirin	Azapropazone
Diflunisal	Dipyron
Salicylates	Isopyrin
	Oxyphenbutazone
<b>Acetic acid derivatives</b>	Phenylbutazone
Etodolac	
Indomethacin	<b>Oxicam derivatives</b>
Sulindac	Lornoxicam
Tolmetin	Meloxicam
Diclofenac	Piroxicam
	Tenoxicam
<b>Fenamates/anthranilic acid derivatives</b>	<b>Coxibs</b>
Flufenamic acid	Celecoxib
Meclofenamic acid	Deracoxib
Mefenamic acid	Firocoxib
Tolfenamic acid	
<b>Propionic acid derivatives</b>	
Carprofen	
Ibuprofen	
Fenoprofen	
Flurbiprofen	
Ketoprofen	
Naproxen	
Suprofen	
Tiaprofenic acid	
<b>Aminonicotinic acid derivatives</b>	
Flunixin meglumine	

The mechanism of action is similar for all drugs in this classification. However, toxicity and pharmacokinetic data vary markedly between the different compounds and species. Cats, for example, are more susceptible to salicylate toxicosis than other species (Roder, 2004a), and dogs are very sensitive to ibuprofen (Rubin and Papich, 1990). Attempts should not be made to extrapolate the therapeutic dose from one species to another (Lees *et al.*, 1991).

Individual factors also affect susceptibility to NSAIDs. Young and aged animals do not have the same metabolic capacity as mature animals. Drug excretion can be delayed in animals with poor hepatic or renal function (Lees *et al.*, 1991; Isaacs, 1996; Roder, 2004a). Dehydration and cardiac disease decrease renal circulation, slowing drug excretion and promoting renal damage. Preexisting gastrointestinal disease could predispose to ulceration when NSAIDs are used.

Various drugs interact with NSAIDs. Changes in bioavailability are by inhibition of absorption, displacement from plasma and tissue binding proteins, and competition for active renal secretion. Some drugs enhance or diminish metabolism of other drugs by hepatic cytochrome P450 enzymes. An incomplete list of possible interactions is provided in Table 28.2. NSAIDs also

TABLE 28.2 Some NSAID–drug interactions. Consult Verbeeck (1990) for more detailed information

<b>Drugs that decrease absorption of some NSAIDs</b>
Aluminum hydroxide
<b>Drugs that compete for protein binding with NSAIDs</b>
Acetazolamide
Corticosteroids
Coumarin
Digitoxin
Hydantoin
Methotrexate
Phenylbutazone
Phenytoin
Salicylates
Sulfonamides
Tolbutamide
Valproic acid
<b>Drugs that increase metabolism rate of some NSAIDs</b>
Antihistamines
Phenobarbital
Phenytoin
Rifampicin
<b>Drugs that decrease metabolism rate of some NSAIDs</b>
Anabolic steroids
Chloramphenicol
<b>Drugs that increase excretion rate of some NSAIDs</b>
Aluminum hydroxide
Magnesium hydroxide
<b>Drugs that decrease excretion rate of some NSAIDs</b>
Probenecid
Methotrexate

affect the bioavailability of other drugs. There have been reports of deaths in people using NSAIDs with methotrexate, and ibuprofen decreases renal clearance of digoxin and lithium. The diuretic effects of furosemide and bumetanide, which are mediated by prostaglandins (PG), are inhibited by NSAIDs. NSAIDs do not influence response to thiazide diuretics in this way, but decrease the hypotensive response. NSAIDs can decrease the effectiveness of angiotensin converting enzyme (ACE) inhibitors such as captopril. NSAIDs have additive effects when used together, with decreased rates of metabolism and clearance for each (Lees *et al.*, 1991; Verbeeck, 1990). NSAIDs also have additive effects when used with corticosteroids, and such mixing should be avoided.

### Toxicity

There are species differences in NSAID tolerance, as noted above. Dogs are more susceptible to ibuprofen and naproxen toxicosis than many other species (Kore, 1990; McKellar *et al.*, 1991; Isaacs, 1996). Use of these drugs in dogs should be avoided.

Ibuprofen doses greater than 5 mg/kg have been associated with adverse reactions in dogs (Villar *et al.*, 1998). Gastric lesions occurred in dogs when 8 mg/kg/day

ibuprofen is given over a 30-day period, either PO or parenterally, though clinical signs were not evident (McKellar *et al.*, 1991; Godshalk *et al.*, 1992; Boothe, 2001; Talcott, 2006). Clinical signs were evident in dogs dosed with 16 mg/kg/d for 8 weeks. Single doses of 50 to 125 mg/kg have been associated with gastrointestinal disease (Jackson *et al.*, 1991; Murphy, 1994; Talcott, 2006) and a perforating gastric ulcer was reported in a dog given a cumulative dose of 110 mg/kg ibuprofen over a period of 48 hours (Godshalk *et al.*, 1992). Doses of 175 to 250 mg/kg have been associated with acute renal failure. Serum ibuprofen concentrations less than 31 µg/mL have not been associated with clinical signs in dogs. Melena was noted with serum concentrations of 138 µg/mL.

Cats dosed with 50 mg/kg ibuprofen had gastrointestinal hemorrhage and irritation (Kore, 1990). Doses of 200 mg/kg were associated with renal failure and over 600 mg/kg caused death. A ferret that had ingested at least 347 mg/kg ibuprofen died from respiratory failure and asystole approximately 12 hours post-exposure (Cathers *et al.*, 2000).

Naproxen induced toxicosis in dogs at a dose of 5 mg/kg/day, and plasma concentrations were >50 µg/mL (Daehler, 1986; Rubin and Papich, 1990; Boothe, 2001). Severe toxicosis was produced in a Samoyed dosed with 5.6 mg/kg naproxen, presumably once per day, over a week. Common clinical signs are anemia, melena and renal and hepatic dysfunction. A perforating ulcer was described in a dog given 10 to 20 mg/kg/day naproxen for several weeks (Daehler, 1986). Doses of 15 mg/kg/day are lethal in some dogs (Daehler, 1986; Gfeller and Sandon, 1991).

Horses do not appear to be sensitive to naproxen toxicosis. Horses given three times the recommended dose of naproxen for 6 weeks did not have clinical signs (Boothe, 2001). Phenylbutazone is commonly used in horses. The recommended oral dose is 4 mg/kg per day (Plumb, 2005). A dose of 4.4 mg/kg every 12 h over a 2-week period was associated with changes of mineralization patterns in cortical bone in growing horses from 18 to 30 months of age. This effect was reversible (Brumbaugh, 2001). The same dose has been associated with other clinical signs including CNS depression and protein-losing enteropathy. Shock was reported in one of nine horses after five days of dosing (Collins and Tyler, 1985). Therapeutic plasma concentrations for phenylbutazone in a horse range from 10 to 30 µg/mL. The therapeutic range in humans, 100 to 150 µg/mL, would be lethal in a horse (Lees *et al.*, 1991). Phenylbutazone doses of 100 mg/kg/day did not cause gastrointestinal ulcers in dogs (Mazué *et al.*, 1982).

### Toxicokinetics

NSAIDs are small, weakly acidic molecules with pK<sub>a</sub> values less than 4.5 and thus are well absorbed in the stomach (Mazué *et al.*, 1982; Kore, 1990; Rubin and

Papich, 1990; Verbeeck, 1990; Isaacs, 1996; Boothe, 2001). Peak plasma concentrations for most NSAIDs occur within 3 hours of oral dosing. Some absorption probably occurs in the proximal small intestine.

Ibuprofen is 60 to 80% bioavailable and naproxen is 68 to 100% bioavailable in dogs (McKellar *et al.*, 1991; Boothe, 2001) and blood concentrations are almost identical whether dosing is PO or IV (Runkel *et al.*, 1972). Naproxen is 50% bioavailable in horses (Boothe, 2001) and nearly 100% bioavailable in pigs (Runkel *et al.*, 1972).

NSAID distribution is variable between individual drugs and animal species. Because they are highly protein bound in the circulation, mostly to albumin, NSAIDs generally have a low volume of distribution (Brater, 1988; Verbeeck, 1990; Boothe 2001; Talcott, 2006). Protein binding in humans is 99% for ibuprofen (Brater, 1988), >99% for naproxen (Runkel *et al.*, 1972; Rubin and Papich, 1990) and 98.7% for ketoprofen (Brater, 1988). The remaining unbound fraction becomes distributed in the extracellular fluid and is responsible for the clinical effects (Brater, 1988; Kore, 1990; Boothe, 2001; Talcott, 2006). Hypoalbuminemia or displacement from protein binding sites by other drugs leads to a transient increase in the unbound fraction. Though this unbound drug is normally rapidly excreted, clinical effects may become evident. NSAIDs can partition into lipid, allowing them to cross cell membranes.

Metabolism of NSAIDs takes place primarily in the liver. Most undergo cytochrome P450 mediated oxidation to increase water solubility. Phenylbutazone is converted to oxyphenbutazone, which is similarly potent, via phase I hepatic metabolism (Lees *et al.*, 1991). Phase I metabolites and, in the case of carboxylic acid group NSAIDs, the parent compound can undergo phase II reactions such as glucuronide conjugation, sulfate conjugation and glutathione conjugation, which further increase water solubility and usually inactivate the drug. Acyl-glucuronide conjugation of propionic acid NSAIDs such as ibuprofen and naproxen is reversible and these drugs deconjugate if excretion is delayed.

Drug elimination is dependent on compound and animal species. Animals less than 6 weeks old and geriatric animals usually metabolize drugs more slowly, and hepatic or renal failure further delays elimination. High plasma protein binding also slows excretion. Plasma clearance is biphasic, with a rapid initial decline as the drug is distributed to the tissues, and then a slower decline as it is metabolized and excreted, terminating drug activity (Lees *et al.*, 1991). Less than 1% of a dose of naproxen or ketoprofen is eliminated unchanged, and approximately 1% of a given dose of ibuprofen is excreted in the urine as the parent compound (Brater, 1988). Urinary excretion is pH dependent and tends to be more rapid in alkali urine due to ion trapping.

Fecal elimination through biliary excretion is important for ibuprofen and naproxen in dogs (Runkel *et al.*,

1972; Gfeller and Sandon, 1991; Isaacs, 1996; Talcott, 2006). Half of a given dose of naproxen is eliminated in the bile of dogs (Runkel *et al.*, 1972). Drugs eliminated in the bile, such as ibuprofen and naproxen, as well as indomethacin, piroxicam, flunixin, tolafenamic acid, meclofenamic acid and diclofenac, undergo enterohepatic circulation in dogs. This prolongs the plasma half-life and is likely the cause of increased susceptibility of dogs to these compounds.

The plasma half-life of ibuprofen is 2.5 to 6 hours in dogs and cats, and 1 hour in rats (Mazué *et al.*, 1982; Lees *et al.*, 1991; Boothe, 2001). The half-life for naproxen in plasma is 35 hours in beagles (Runkel *et al.*, 1972), 74 hours in mixed-breed dogs (Isaacs, 1996), 5 hours in horses (Lees *et al.*, 1991; Isaacs, 1996), 4.8 hours in cows (Lees *et al.*, 1991), 4.8 hours in minipigs, 1.9 hours in rhesus monkeys and 8.7 hours in guinea pigs. Elimination of phenylbutazone in dogs appears to follow zero-order kinetics (Lees *et al.*, 1991). The plasma half-life for phenylbutazone is between 5 and 8 hours in horses, 37 hours in cattle and 4 hours in swine.

### Mechanism of action

The basic mechanism of action of NSAIDs is inhibition of cyclooxygenase (COX) enzymes. These enzymes are found in all cells except mature erythrocytes (Kore, 1990; Boothe, 2001). Arachidonic acid, a 20-carbon unsaturated fatty acid, is released from the cell membrane by phospholipase A<sub>2</sub> and phospholipase C when a cell is damaged. Carprofen causes moderate inhibition of these phospholipases (Lees *et al.*, 1991; McKellar *et al.*, 1991). Though arachidonic acid itself has little activity, it can enter two pathways: the COX pathway, which produces eicosanoids, or the lipoxygenase pathway, which produces leukotrienes. Oxidation of arachidonic acid by COX and further metabolism by other enzymes leads to the production of various prostaglandins (PGs) and release of oxygen free radicals (Lees *et al.*, 1991; Boynton *et al.*, 1998). These PGs include PGH<sub>2</sub>, PGE<sub>2</sub> and PGI<sub>2</sub>. With the addition of prostacyclin synthase, PGF<sub>2α</sub> is formed, and thromboxane synthase is needed for production of thromboxane A<sub>2</sub>.

PGE<sub>2</sub> and PGI<sub>2</sub> have similar actions, though the effects of PGI<sub>2</sub> tend to be of shorter duration. PGE<sub>2</sub>, which is secreted by the gastrointestinal mucosa and at other sites, causes smooth muscle relaxation and vasodilation, which enhances blood flow to the kidneys and gastric mucosa, and increases vascular permeability. It inhibits gastric acid production, inhibits pepsin production, increases gastric mucus synthesis and is believed to mediate repair and turnover of gastric epithelium (Collins and Tyler, 1985; Boynton *et al.*, 1988; Wallace *et al.*, 1990). PGE<sub>2</sub> is also found in inflammatory exudate and enhances pain response due to bradykinin and histamine. PGI<sub>2</sub> also inhibits platelet aggregation.

NSAIDs bind the active site of COX, usually through competitive inhibition, though aspirin binds platelet COX irreversibly (Rubin and Papich, 1990). There are two isoforms of COX, designated COX<sub>1</sub> and COX<sub>2</sub>. COX<sub>1</sub> is found in almost all tissues, including the gastrointestinal tract, platelets, endothelium and kidneys, is continuously produced and functions in tissue homeostasis. Most of the adverse effects associated with NSAID use are due to inhibition of COX<sub>1</sub>. Inhibition of PGE<sub>2</sub> promotes production of gastric acid and pepsin, and decreases the ability of the mucosa to secrete mucus glycoproteins and bicarbonate and respond to injury. Impairments to mucosal circulation due to loss of PG activity produce mucosal hypoxia and thrombosis.

Loss of the vasodilative actions of PGE<sub>2</sub> and PGI<sub>2</sub> in the kidneys through inhibition of COX<sub>1</sub> leads to hypoxic renal injury (Isaacs, 1996; Rubin and Papich, 1990). Production of PGs by the kidneys is relatively low, and the renal effects of acute NSAID toxicosis are seldom reported in the veterinary literature (Rubin and Papich, 1990; Talcott, 2006). Renal pathology is more often associated with chronic NSAID use. Renal papillary necrosis is frequently seen in horses, and is often associated with chronic phenylbutazone use (Gunson, 1983; Rubin and Papich, 1990). This lesion is also reported in cats, dogs, mice, rats, gerbils, hamsters, rabbits, desert mice, primates and pigs (Brix, 2002). Renal failure in cats administered meloxicam first parenterally and then orally has been recently reported (Dyer *et al.*, 2009).

Recent evidence suggests that low doses of NSAIDs cause degeneration of medullary interstitial cells and later damage to vascular endothelium, leading to microvascular thrombosis and hypoxia. Higher doses produce more rapid endothelial damage (Brix, 2002). Dehydration is a major predisposing factor for renal papillary necrosis. Dehydration commonly occurs with gastrointestinal disease, diuretic use, anesthesia, surgical stress, hemorrhagic shock or sepsis. Other risk factors described for dogs include advancing age, congestive heart failure, hepatic cirrhosis, preexisting renal problems, hypotension and concurrent administration of nephrotoxic drugs such as gentamicin or amphotericin.

COX<sub>2</sub> is produced by macrophages, fibroblasts, chondrocytes, endothelial cells and some other cell types (Roder, 2004a). This isoform only functions intermittently and is induced by cytokines in areas of inflammation (Isaacs, 1996; Roder, 2004a; Talcott, 2006). Inhibition of this enzyme produces antipyretic, analgesic and anti-inflammatory effects of NSAIDs.

Most NSAIDs inhibit both isoforms of COX, but a new class of COX<sub>2</sub>-specific NSAIDs was developed as a safer alternative. These drugs include celecoxib, deracoxib, rofecoxib, diclofenac, etodolac, firocoxib, flosulide and meloxicam. However, rofecoxib (Vioxx®) was voluntarily withdrawn from the market in 2004 due to increased risk



of adverse cardiovascular events in humans, and controversies still exist whether other compounds of this class pose a similar risk. Selectivity is species specific, and thus these new drugs sometimes cause COX<sub>1</sub> inhibition in domestic species (Talcott, 2006). Dogs treated with deracoxib and firocoxib did have fewer clinical signs and gastric lesions than dogs treated with other NSAIDs in one study (Senello and Leib, 2006). Interestingly, carprofen is COX<sub>2</sub> specific in dogs but not in humans (Talcott, 2006).

Leukotrienes (LTs) are also produced from arachidonic acid via the lipoxygenase pathway. 5-Lipoxygenase produces LTA<sub>4</sub> which is converted to LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub> and others. LTB<sub>4</sub> is an important chemoattractant for neutrophils. Various LTs cause vasoconstriction, bronchospasm and increased vascular permeability (Boynton *et al.*, 1988; Strøm and Thomsen, 1990; Lees *et al.*, 1991). Some NSAIDs inhibit lipoxygenase. It has been found that in dogs, flunixin is a more potent inhibitor of LTB<sub>4</sub>-mediated neutrophil migration than phenylbutazone, which is more potent than indomethacin. Alternately, some NSAIDs actually increase LT production due to increased availability of arachidonic acid not entering the COX pathway.

NSAIDs can also inhibit phosphodiesterase. Phosphodiesterase breaks down cyclic AMP (cAMP). Increased intracellular cAMP can stabilize lysosomal membranes in polymorphonuclear leukocytes, inhibiting release of inflammatory products (Kore, 1990). Other effects of NSAIDs include inhibition of phosphatidylinositol 3'-kinase Akt signaling, important in cytokine pathways and cell regulation, mitogen-activated protein kinases involved in cell regulation, peroxisome proliferator-activated receptors, NF $\kappa$ B and heat shock proteins involved in transcription. NSAIDs also uncouple oxidative phosphorylation and thus inhibit cellular energy production (Little *et al.*, 2007).

Some NSAIDs have topical irritant properties. As weak acids, they partition to the gastric mucosa leading to decreased hydrophobicity of mucus and thinning of the mucus barrier, allowing gastric acid to penetrate to the epithelial layer. NSAIDs frequently cause mild and transient liver damage associated with cholestasis and increased liver enzymes (Boynton *et al.*, 1988; Isaacs, 1996; Roder, 2004a). More severe problems, such as hepatic necrosis, are rare. Hepatotoxicity is uncommon with ibuprofen in dogs. More commonly reported in dogs is idiosyncratic hepatotoxicosis due to carprofen (Albretsen, 2002; Roder, 2004a; Talcott, 2006). Approximately one third of these cases were in Labrador retrievers.

Cartilage degeneration has been reported in mice given aspirin, phenylbutazone, indomethacin, ibuprofen and naproxen (Isaacs, 1996). Anaphylactic reactions have been reported, but hypersensitivity to NSAIDs is rare. This problem is less common in domestic animals, but

has been documented in dogs due to phenylbutazone administration (Rubin and Papich, 1990; Lees *et al.*, 1991; Isaacs, 1996).

### Clinical signs

The most common clinical signs of NSAID overdose in all species are due to gastric mucosal irritation and ulceration. NSAIDs are capable of producing peptic ulcers in animal models (Carson and Strom, 1988). Clinical signs do not always correlate well with the severity of the lesion, probably due to the analgesic effect of NSAIDs. The asymptomatic ulcer rate among NSAID users is estimated at 50% (Isaacs, 1996). Emesis is frequently observed and the vomitus sometimes contains blood. Anorexia, diarrhea, melena and abdominal pain or colic are often noted. Animals with gastric perforations usually present with a distended abdomen due to effusion, dehydration, pyrexia, collapse, pallor and abdominal pain (Godshalk *et al.*, 1992; Talcott, 2006).

Lower gastrointestinal tract effects can include protein-losing enteropathy, most common in horses, and stricture formation. Horses with hypoproteinemia often have secondary edema (Collins and Tyler, 1984, 1985). Endotoxemia and shock have been noted in horses secondary to gastrointestinal ulceration.

Analgesic nephropathy occurs in humans and has been reported in dogs given naproxen and more recently in cats on meloxicam therapy (Isaacs, 1996; Boothe, 2001; Dyer *et al.*, 2009). Chronic use of NSAIDs in high doses appears to be a predisposing factor. Clinical signs are evident after 2 weeks or many months of chronic dosing or, sometimes, after single very high doses. Acute onset of oliguric renal failure has been described (Kore, 1990; Villar *et al.*, 1998; Roder, 2004a). Animals may present with polyuria and polydipsia, dehydration, oral ulceration and uremic coma (Mazué *et al.*, 1982).

Hepatotoxicosis is not common in NSAID-intoxicated small animals, though it has been reported in a dog given naproxen (Kore, 1990; Lees *et al.*, 1991; Isaacs, 1996) and as an idiosyncratic reaction to carprofen (Albretsen, 2002). Clinical signs include anorexia and weight loss, vomiting, lethargy, icterus, ascites and coagulopathy. Increased bleeding times are rarely reported in domestic animals (Murphy, 1994). Prolonged gestation and teratogenesis have been seen experimentally (Lees *et al.*, 1991).

### Clinical chemistry

Approximately one quarter of gastroduodenal ulceration cases in dogs present with microcytic hypochromic anemia due to chronic blood loss (Stanton and Bright, 1989). Acute blood loss is uncommon. Individuals with acute blood loss present with rapid deterioration, normocytic and normochromic anemia, a normal albumin to globulin ratio and evidence of regeneration (Stanton and Bright,



1989; Wallace *et al.*, 1990). Peracute hemorrhage is rare and occurs if a mesenteric blood vessel is eroded in the area of ulceration. Such a complication would be rapidly lethal and not allow time for a regenerative response. Increased PCV has been seen in horses with dehydration secondary to diarrhea (Collins and Tyler, 1985).

Fecal occult blood is unreliable. It can be negative for low-level blood loss or positive if red meat was fed within 72 hours of testing (Talcott, 2006). Hypoalbuminemia has been observed with gastrointestinal ulcers and protein-losing enteropathy secondary to NSAID use. Both albumin and globulin concentrations are decreased. Coagulation profiles reveal poor platelet aggregation, thrombocytopenia and increased bleeding times.

Serum chemistry reveals increased ALT, AST, ALP and bilirubin (Albretsen, 2002; Roder, 2004a). A mild, transient rise in liver enzymes often occurs during the first week of NSAID administration. Elevated blood urea nitrogen (BUN) indicates nephropathy. Phosphorus, calcium and potassium are usually also elevated. Animals with renal damage due to NSAIDs present with isosthenuria, hematuria and proteinuria. Ketonuria and pyuria have also been reported. Horses on chronic high-dose phenylbutazone therapy had normal lymphocyte counts with a left shift due to neutrophil loss into the gastrointestinal tract. Increased serum glucose concentrations were attributed to endotoxemia. Hypokalemia and hyponatremia are commonly seen in horses with diarrhea (Collins and Tyler, 1985).

### Diagnosis and management

Diagnosis of NSAID toxicosis is often based on history and clinical signs. Serum or plasma testing for NSAID drugs is available at many veterinary laboratories and human hospitals. Serum drug concentrations are unlikely to be useful in management, but some reference values are listed in the toxicokinetics portion of this section.

Endoscopy is the most sensitive test for gastric ulceration, though occasionally the ulcer will not be visible. Anesthesia is required, thus the patient must be stabilized. Survey radiographs are not usually useful. If there is gastrointestinal perforation, then poor visualization of serosal surfaces, peritoneal effusion, free gas in the peritoneal cavity and intestinal ileus are often noted (Godshalk *et al.*, 1992; Talcott, 2006). Large ulcerations have been identified on contrast radiographs. However, if perforation is present, barium sulfate will complicate peritonitis, thus a water soluble agent is preferred. Ulcers can be detected using ultrasound in an animal given water by stomach tube. The gastric wall is thickened with a loss of the normal five-layer structure, disruption of the mucosa and gas bubble formation. Increased fluid in the abdomen is seen in the case of peritonitis due to perforation.

Renal papillary damage can be diagnosed in humans, horses and foals using ultrasound (Brix, 2002; Roder, 2004a). Computed tomography has been used to diagnose renal papillary necrosis in humans, and magnetic resonance imaging has been used experimentally (Brix, 2002).

Animals on NSAID therapy should be closely monitored for adverse effects. Attitude, appetite, hydration, electrolyte status, urine concentrating ability, urine protein concentrations, BUN, creatinine and liver enzymes can be examined (Villar *et al.*, 1998; Roder, 2004).

When animals present with adverse effects attributable to NSAIDs, administration of the drug should be discontinued. Gastric emptying is appropriate for animals that have ingested large quantities of NSAIDs but are not yet showing clinical signs (Kore, 1990). Emetics are sometimes used for large single ingestions, though they are contraindicated in animals with severe CNS suppression. Gastric lavage can be performed in the anesthetized, intubated animal (Cathers *et al.*, 2000). Activated charcoal and cathartics are given after gastric emptying or if the animal presents later. Repeated activated charcoal administration is recommended for various reasons. Some tablets are sustained release, and regular tablets can form concretions in the stomach and have delayed absorption. Furthermore, activated charcoal prevents enterohepatic cycling which is the cause of a prolonged half-life of ibuprofen and other NSAIDs in dogs.

Animals should be monitored for hydration, electrolyte imbalances, acid-base imbalances, blood glucose, liver enzymes, BUN and creatinine, body temperature and blood pressure. Deviations from expected values can be corrected as needed. Fluid therapy is needed to correct dehydration, hypotension, electrolyte imbalances and improve renal perfusion. It has been suggested that fluids be given at twice maintenance rates. Villar *et al.* (1998) recommend 0.9% saline, or 0.45% saline plus 2.5% dextrose in hypoglycemic animals, given at a rate of 120 mL/kg/day plus estimated fluid losses, over a 48- to 72-hour period. Lactated Ringers solution can contribute to electrolyte imbalances and thus should be used with caution. Dopamine at a dose of 2.5 µg/kg/minute or dobutamine at 2.5 mg/kg/minute is given to increase renal perfusion (Kore, 1990). Diuretics can predispose animals to dehydration and subsequent renal hypoperfusion and thus should be avoided (Albretsen, 2002). Sodium bicarbonate is used to treat acidosis (Murphy, 1994; Roder, 2004a). Urine alkalization for ion trapping is sometimes useful with salicylates, aspirin and ibuprofen, but urinary pH must exceed 7.5 to 8 to increase naproxen secretion. Urine alkalization must be used with caution to prevent metabolic alkalosis.

Gastric lavage with iced saline or water has been recommended for severe and continued gastric bleeding, but the efficacy of this treatment is questionable and surgical correction is often required in cases of refractory

bleeding (Wallace *et al.*, 1997). Severe anemia is treated with transfusions or use of blood substitutes (Murphy, 1994; Sellon, 2006). Acute loss of greater than 30% of the blood volume, a PCV less than 20%, plasma protein less than 3.5 g/dL or continued blood loss are possible indications for transfusion (Talcott, 2006). Vitamin K<sub>1</sub> is given to aid coagulation (Kore, 1990). Perforating ulcers must be managed surgically.

Gastrointestinal protectants are typically used in NSAID overdose cases to prevent or heal gastric ulcerations. Gastric pH must be maintained above 3 for 18 to 20 hours a day for effective healing, and above 4 in critically ill patients. Gastric pH above 4 inhibits fibrinolysis and the activity of pepsin. Gastric pH must be above 6 to promote platelet aggregation and prevent clot dissolution, therefore allowing hemostasis. Gastric secretion is variable in beagles, which have a median pH of 1.30 under normal conditions and 4.44 if fasted (Bersenas *et al.*, 2005). Therefore, simply withholding food has the benefit of decreasing gastric acid secretion and minimizing emesis.

Treatment with antacids such as magnesium hydroxide (milk of magnesia) or aluminum hydroxide is probably adequate for animals exposed to low NSAID doses. Antacids require dosing every 2 to 4 hours and sometimes produce changes in fecal consistency (Wallace *et al.*, 1990). Products containing bismuth subsalicylate (Pepto Bismol and Kaopectate) should be avoided because salicylate is an NSAID.

Misoprostol is a long-lasting synthetic analog of PGE<sub>1</sub>. It is the only agent that consistently prevents and treats NSAID-induced gastric and duodenal ulcers (Isaacs, 1996). When given concurrently with aspirin, misoprostol prevented gastric ulcers in dogs (Villar *et al.*, 1998). Dogs given NSAIDs and misoprostol had less gastrointestinal hemorrhage, less vomiting and less mucosal ulceration on endoscopy than dogs given NSAIDs only (Talcott, 2006). Misoprostol is believed to protect renal function in domestic animals and be chondroprotective in pigs (Isaacs, 1996). At high doses, misoprostol can cause diarrhea and use of this synthetic prostaglandin is contraindicated in pregnant animals (Villar *et al.*, 1998). Dogs may be given 1 to 5 µg/kg misoprostol PO every 8 hours (Plumb, 2005).

Proton pump inhibitors suppress gastric acid secretion by inhibiting the H<sup>+</sup>/K<sup>+</sup>-ATPase pump in gastric parietal cells (Walan *et al.*, 1989; Villar *et al.*, 1998). Omeprazole and pantoprazole are proton pump inhibitors (Bersenas *et al.*, 2005). Omeprazole has been used successfully in humans and dogs to treat gastric ulcer. Bersenas *et al.* (2005) found 2 mg/kg omeprazole PO given to dogs twice per day kept their gastric pH from going below 3 for 90.9% of the day, and kept the gastric pH above 4 for 78.3% of the day, though one in six dogs vomited. Omeprazole remains biologically

active for nearly 24 hours and only need be given once daily. Plumb (2005) lists the canine daily dose as 0.5 to 1.0 mg/kg PO, and the feline dose as 0.7 mg/kg. Villar *et al.* (1998) recommend a 3- to 4-week treatment protocol. Rebound acid secretion has been reported at discontinuation of omeprazole therapy (Driman *et al.*, 1995).

Sucralfate is an aluminum salt of sucrose sulfate. This drug complexes with exposed proteins on the surface of gastric ulcers, specifically fibrinogen and albumin, and acts as a direct mucosal protectant. Sucralfate has five times as much affinity for damaged mucosa as it does for normal mucosa (Villar *et al.*, 1998). Sucralfate also adsorbs pepsin and bile acids. This drug is believed to promote bicarbonate and mucus secretion by the gastric mucosa. Sucralfate is equally as effective as cimetidine at reducing discomfort and promoting ulcer healing. Dogs given sucralfate to treat gastric ulcers, with and without concurrent cimetidine treatment, had completed healing of the gastric mucosa within nine days of treatment. Sucralfate can be used alone if gastric hyperacidity is not suspected (Wallace *et al.*, 1990). If used with cimetidine, dosing should be staggered or cimetidine should be given by a parenteral route. Sucralfate interferes with absorption of cimetidine from the gastrointestinal tract (Wallace *et al.*, 1990). Cimetidine should be given 1 hour before a meal. Dogs are dosed every 8 hours and dogs weighing >20 kg are given 1 g per dose, and smaller dogs are dosed with 0.5 g (Murphy, 1994; Albrechtsen, 2002; Plumb, 2005). Cats are given a total of 0.25 to 0.5 g every 8 to 12 hours and ferrets are given 75 mg/kg four to six times a day, preferably 10 minutes before feeding. Foals are given 1 to 2 grams PO two to three times daily.

H<sub>2</sub> histamine receptor antagonists, including cimetidine, ranitidine and famotidine, are routinely used to treat NSAID-induced gastric ulcers. Histamine stimulates gastric parietal cells to secrete hydrogen ions through an H<sup>+</sup>/K<sup>+</sup>-ATPase pump. Acetylcholine, gastrin and histamine stimulate this proton pump. H<sub>2</sub> inhibitors only block the histaminic effect on parietal cells, but cause significant inhibition of gastric acid secretion nonetheless (Bersenas *et al.*, 2005). H<sub>2</sub> inhibitors are likely to suppress clinical signs associated with unhealed ulcers (Talcott, 2006).

Cimetidine has been commonly used to treat and prevent NSAID-induced ulcers. Cimetidine is believed to decrease gastrointestinal discomfort and does decrease gastric acid secretion (Boulay *et al.*, 1986; Isaacs, 1996). Cimetidine has not been proven to be effective with continued NSAID use and did not prevent gastric ulcer formation when used concurrently with aspirin. Cimetidine is more useful when NSAID administration has ended and could be helpful if given after a single large dose (Kore, 1990). Gastric acid production is dependent on local circulation, which provides oxygen and energy. Cimetidine decreases gastric blood flow, which

contributes to decreased mucosal alkalinity (Cheung and Sonnenschein, 1983). Reduced circulation also contributes to tissue hypoxia. Cimetidine is known to inhibit hepatic cytochrome P450 enzymes and therefore could decrease the rate of NSAID metabolism (Talcott, 2006; Wallace *et al.*, 1990; Verbeeck, 1990). Cimetidine given at a dose of 6mg/kg every 6 hours caused decreased basal acid output in dogs by 30 to 50% and food induced output by 63 to 71%. When 12mg/kg was used every 6 hours, there was a 70 to 80% decrease in basal acid output and complete suppression of food-induced output (Boulay *et al.*, 1986). Cimetidine is given to dogs at a dose of 5 to 10mg/kg PO, SC, or slow IV every 6 to 8 hours (Villar *et al.*, 1990; Plumb, 2005). Similar dosing protocols are used in cats and ferrets.

Ranitidine does not inhibit microsomal enzymes like cimetidine. However, Bersenas *et al.* (2005) found no significant effect on gastric acid secretion in dogs dosed with ranitidine. Ranitidine also decreases gastric blood flow. Ranitidine is given at a dose of 0.5 to 2.0mg/kg PO, IV, or IM every 8 to 12 hours in dogs, and 2.5mg/kg IV or 3.5mg/kg PO in cats every 12 hours (Plumb, 2005). Villar *et al.* (1998) recommend continuous treatment for 3 to 6 weeks.

Famotidine increases intragastric pH in dogs better than ranitidine. However, a 0.5mg/kg dose of famotidine given two to three times a day did not result in a prolonged gastric pH increase (pH > 4) in dogs (Bersenas *et al.*, 2005). Famotidine was found protective against reduced gastric perfusion when used at a dose of 0.5mg/kg IV in dogs given diclofenac (Hata *et al.*, 2005). Dogs are given 0.5 to 1mg/kg famotidine PO or slow IV every 12 hours. The dose for cats is 0.5mg/kg and 0.25 to 0.5mg/kg is the dose for ferrets. Horses are given 0.23mg/kg IV or 1.88mg/kg PO every 8 hours, 0.35mg/kg IV or 2.8mg/kg PO every 12 hours.

The prognosis for NSAID toxicosis is dependent on chronicity, dose and clinical signs (Talcott, 2006). A study by Wallace *et al.* (1990) found that seven out of seven dogs treated for chronic NSAID toxicosis recovered after two to nine days of hospitalization. The mean hospital stay was six days. Gastrointestinal irritation and ulceration are reversible, but perforation and peritonitis require intensive surgical and medical management and have a guarded prognosis. Renal effects such as nephropathy are often reversible, but papillary necrosis is a permanent change, though well tolerated in horses. Loss of the long loops of Henle decreases urine concentrating ability, but horses with renal papillary necrosis usually appear clinically normal (Gunson, 1983; Roder, 2004a). Severe acute cortical necrosis is associated with irreversible renal failure. NSAID-induced coagulopathies are reversible once the NSAID has been eliminated. Dogs with idiosyncratic hepatic injury usually recover within 4 weeks (Albretsen, 2002).

### Postmortem findings

NSAID toxicosis is most commonly associated with gastrointestinal lesions in domestic animals. Mild lesions include mucosal edema, irritation and petechiation, which progress to erosive and then ulcerative lesions. The location for gastric ulceration in the canine stomach is variable; lesions can be near the pylorus, lesser curvature of the fundus or diffuse. Duodenal ulcers and perforations have been reported in dogs, but can be influenced by predisposing factors. Perforations are reported to occur in the stomach, small intestine or colon. Ulcers that erode mesenteric vasculature are rapidly lethal.

Gastric ulcerations in the horse usually occur in the glandular mucosa adjacent to the margo plicatus (Collins and Tyler, 1984, 1985; Roder, 2004a). Linear circular erosions were reported in the duodenum of horses dosed with phenylbutazone, and ulcers and erosions were found throughout the small intestines. Ulcerative colitis of the right dorsal colon is commonly seen in horses with NSAID toxicosis. Fibrinonecrotic typhlocolitis was reported by Collins and Tyler (1985).

Papillary necrosis occurs with long-term NSAID administration (Mazué *et al.*, 1982). The lesion is bilateral and the papillae are cavitated, yellow-green to orange and demarcated from the medulla by hemorrhage. The lesion is most severe at the poles. Microscopically, the papilla has undergone coagulative necrosis with dilation of the collecting ducts and loops of Henle. Interstitial fibrosis extends through the medulla and cortex in chronic cases (Gunson, 1983). This lesion has been reported in horses, dogs, cats, mice, rats, gerbils, hamsters, rabbits, desert mice, primates and pigs (Brix, 2002). The lesion is common in horses given phenylbutazone, but has also been reported with flunixin, aspirin and dipyrone (Gunson, 1983). Papillary necrosis occurred in two of five dogs dosed with piroxicam (Talcott, 2006).

Interstitial nephritis, with multifocal or diffuse infiltrates of lymphocytes, has been reported, as has vacuolar degeneration of proximal and distal convoluted tubules (Kore, 1990). Tubular nephritis with epithelial necrosis and regeneration has also been described (Mazué *et al.*, 1982). Acute cortical necrosis due to NSAID toxicosis has been documented (Jones *et al.*, 1992).

Lymphoid necrosis has been noted in dogs and a ferret. Mild necrosis in the white pulp of the spleen was described in the ferret (Cathers *et al.*, 2000). Depletion and necrosis of germinal centers was discovered in a dog, though circulating lymphocytes were within the reference range.

### Aspirin and other salicylates

This group, which constitutes the most extensively used over-the-counter drugs, includes acetylsalicylic acid, or



aspirin, sodium salicylate, bismuth subsalicylate and diflunisal. Aspirin and salicylates are NSAIDs with many characteristics similar to those of other NSAIDs, as described previously. Aspirin and salicylate also have certain unique properties. Clinical toxicosis of products containing bismuth subsalicylate, an over-the-counter drug commonly used for gastrointestinal distress, are similar to those associated with other sources of salicylate, thus these products will be further discussed in this section.

Salicylates have been used since ancient times by Greek and Roman physicians Hippocrates and Galen, by South African tribes and by North American Indians. Reverend Edmund Stone wrote of the use of *Salix alba* bark in the journal of the Royal Philosophical Society in London in 1763. Salicylic acid was first isolated from the bark of willows (*Salix* spp.) and beech trees (*Fagus* spp.) in the early 19th century (Lees *et al.*, 1991). Bayer Pharmaceutical Company synthesized acetylsalicylic acid in 1893 and aspirin has been marketed since 1899.

Contraindications for aspirin use include coagulation disorders, recent (within 1 week) surgery and concurrent use with certain drugs. See Table 28.2 for a list of drugs that may interact with aspirin and other NSAIDs. Aspirin inhibits the diuretic effects of spironolactone (Verbeeck, 1990).

### Toxicity

The efficacy of aspirin is dose dependent. Aspirin is used in dogs at doses of 10 to 25 mg/kg every 8 to 12 hours for analgesia. Dogs dosed with 25 mg/kg aspirin every 8 hours for up to 3 weeks had no serious adverse effects and peak plasma concentrations were  $>50\mu\text{g/mL}$  in mature dogs (Lees *et al.*, 1991). The half-life is likely to be increased in pups less than 30 days old due to limited metabolic capability (Waters *et al.*, 1993). A study of dogs dosed with 25 to 35 mg/kg aspirin every 8 hours found increased fecal hemoglobin and gastric ulcerations were visualized on endoscopy (McKellar *et al.*, 1991). Seizures were reported on a dog given 37 mg/kg aspirin every 12 hours for 2 weeks (Schubert, 1984). Clinical signs in dogs dosed with 50 mg/kg aspirin every 12 hours included emesis. Similar daily doses were associated with perforating gastric ulcers within 4 weeks (Kore, 1990). One dose of 400 mg/kg caused hemorrhage and daily dosing caused pyloric ulcers within 2 weeks (Mazué *et al.*, 1982).

Cats are more susceptible to the toxic effects of aspirin than dogs and humans (Papich, 1990; Roder, 2004a). Doses of 25 mg/kg every two to three days are recommended to control thromboembolic disease and 10 mg/kg every other day can be used for analgesia and antipyresis (Plumb, 2005). A dose of 10.5 mg/kg aspirin every 52 hours produced plasma concentrations ranging from 50 to  $20\mu\text{g/mL}$ . No clinical signs were seen in cats dosed with 25 mg/kg aspirin every 48 hours and

no severe signs were seen in cats dosed with 25 mg/kg aspirin every 24 hours for 2 to 3 weeks (Lees *et al.*, 1991; Boothe, 2001). Aspirin doses between 100 and 110 mg/kg daily have caused death in cats within seven days (McKellar *et al.*, 1991). Young cats, old cats or cats with renal or hepatic disease are believed to be particularly sensitive (Kore, 1990).

Aspirin can be used in cattle and goats, but they require larger oral doses to reach appropriate plasma concentrations. A dose of 100 mg/kg PO given every 12 hours in cattle maintains a therapeutic concentration of  $30\mu\text{g/mL}$  (Davis, 1980; Boothe, 2001). IV dosing with 6.5 mg/kg hourly is associated with plasma concentrations of 50 to  $20\mu\text{g/mL}$ , 26 mg every 1.6 hours yields plasma concentrations of 200 to  $50\mu\text{g/mL}$ , and 39 mg every 2 hours yields concentrations of 300 to  $50\mu\text{g/mL}$  in the plasma. Horses can be given 35 mg/kg aspirin IV according to Davis (1980). However, a single oral dose of 20 mg/kg was associated with prolonged bleeding times (Boothe, 2001).

### Toxicokinetics

Aspirin is lipophilic at acid pH, thus it is readily absorbed from the stomach and duodenum in dogs and cats (Rubin and Papich, 1990; Talcott, 2006). Aspirin is 68 to 76% bioavailable in dogs, with some variation based on the formulation (Boothe, 2001). Enteric-coated and buffered products are used in humans and dogs (McKellar *et al.*, 1991; Murtaugh *et al.*, 1993; Boothe, 2001). Buffered aspirin is more soluble and less ionized, thus slowing absorption and decreasing gastric irritation. Acid-resistant forms are more readily absorbed at the relatively alkaline pH range of the duodenum and absorption is delayed up to 12 hours (Verbeeck, 1990). Ingestion of a fatty meal can reduce aspirin bioavailability by 30% (Mazué *et al.*, 1982). Aspirin is 70% bioavailable in cattle, but absorption is relatively slow. Salicylates in bismuth subsalicylate are 97.5% absorbed in dogs and 85.4% absorbed in cats (Papich *et al.*, 1987).

Peak serum concentrations occur 4 hours after oral dosing with buffered aspirin. Peak serum concentrations are lower in immature dogs than in mature dogs after PO or IV dosing. Circulating salicylate is 72% protein bound in humans and 45% protein bound in dogs. There is more free, and therefore bioavailable, salicylate in the hypoalbuminemic animal, but the excess is rapidly eliminated (Boothe, 2001). Aspirin is rapidly distributed to most tissues, including synovial fluid, peritoneal fluid, saliva and milk. Aspirin has a lower volume of distribution in cattle compared to other domestic species (Davis, 1980).

Aspirin is rapidly hydrolyzed to salicylate by esterases in the gastrointestinal tract, liver and erythrocytes. These enzymes are less efficient in immature dogs



(Waters *et al.*, 1993). Most of the metabolism takes place in the liver. Salicylate is bioactive and responsible for most of the clinical effects of aspirin. Salicylate is conjugated to glucuronide or glycine. The glucuronide pathway is deficient in cats, limiting their elimination of salicylate. Bismuth subsalicylate is metabolized to bismuth and salicylate in the gastrointestinal tract (Boothe, 2001).

The kidneys eliminate salicylate and its glycine conjugate (Boothe, 2001). Two to 30% of a dose of aspirin is excreted as unconjugated salicylate (Verbeeck, 1990). The rate of elimination for aspirin varies with species and age (McKellar *et al.*, 1991). The elimination half-life in dogs is dependent upon the dose form. The half-life for enteric-coated products ranges from 7.5 to 12.2 hours. The elimination half-life for aspirin given IV is 2.2 to 8.7 hours (Boothe, 2001). The elimination half-life in cats is long and increases with dose. If low doses of 5 to 12 mg/kg are given, the elimination half-life is 22 to 27 hours, but the half-life for the much higher dose of 25 mg/kg is 45 hours. Salicylate is considered to have zero-order kinetics in cats (Lees *et al.*, 1991). The elimination half-life in horses is about an hour due to ion trapping in alkaline urine. Salicylates are normal components of equine urine (Boothe, 2001). The elimination half-life of salicylates in cattle is about half an hour (Lees *et al.*, 1991; Boothe, 2001). Young animals metabolize aspirin more slowly, and the elimination half-life may be prolonged in puppies less than 30 days old, piglets, kids and foals.

### Mechanism of action

The effects attributed to aspirin are mostly caused by the active metabolite salicylate (McKellar *et al.*, 1991). Salicylate inhibits COX to block PG synthesis, as described for NSAIDs. Other NSAIDs competitively inhibit COX, but salicylates permanently inactivate the enzyme by acetylating a serine residue (Kore, 1990; Rubin and Papich, 1990; Boothe, 2001; Talcott, 2006). Platelets are unable to synthesize COX. Unlike other NSAIDs, where coagulopathy resolves once the drug is eliminated, it takes about a week to resolve aspirin-induced coagulopathy.

Aspirin has a direct irritant effect on the gastric mucosa. Aspirin is an acidic drug and is taken up by the mucosal epithelium, where it becomes concentrated (Carson and Strom, 1988; Rubin and Papich, 1990; Isaacs, 1996). Aspirin inhibits oxidative phosphorylation of the gastric mucosal epithelium, thus decreasing ATP production and  $\text{Na}^+/\text{K}^+$  movement across the cell membrane and producing cell swelling and necrosis (Rubin and Papich, 1990). Disruption of ion transport allows increased  $\text{H}^+$  ion back-diffusion into the mucosa. Injury can extend into submucosal capillaries, leading to hemorrhage, inflammation and ulceration. IV dosing of cats produced gastric erosions and ulcers, confirming that

there is also a role for COX inhibition and reduced PG synthesis in the pathophysiology of gastric mucosal damage (Villar *et al.*, 1998).

Aspirin uncouples oxidative phosphorylation, as noted above. Aspirin allows penetration of  $\text{H}^+$  across the mitochondrial membrane, thus disrupting the proton gradient. The result is inhibition of the enzyme ATP synthetase. Energy that would go into ATP production is dissipated as heat. When oxidative phosphorylation is inhibited systemically, the result is elevated body temperature (Roder, 2004a).

Salicylates can produce acidosis and increase the anion gap. Salicylate and metabolites thereof increase the anion gap directly (Schubert, 1984; Kore, 1990). Anaerobic metabolism compensates for the reduced ATP production when oxidative phosphorylation is inhibited, producing metabolites such as lactic acid, pyruvic acid and ketones, contributing to acidosis. Aspirin is also believed to directly stimulate respiratory centers in the CNS, causing hyperventilation and respiratory alkalosis, and promoting renal secretion of bicarbonate (Kore, 1990; Boothe, 2001). Later in the progression of the toxicosis, CNS suppression can decrease the respiratory rate, also contributing to acidosis.

### Clinical signs

Similar to other NSAIDs, gastric irritation is the most common side effect of aspirin. Vomiting, gastric ulceration and hemorrhage are often observed. Depression is also seen in aspirin toxicosis in dogs and cats (Kore, 1990; Jones *et al.*, 1992). Profound depression has been reported in dogs given high doses of aspirin. Dogs collapse or appear too weak to stand. Restlessness progressing to tremors, seizures and eventually coma has been observed in dogs (Kore, 1990). One dog had a progressive increase in seizure duration from 5 to 45 minutes (Schubert, 1984). Seizures and coma have also been observed in cats (Jones *et al.*, 1992; Isaacs, 1996). Seizures have been attributed to hypoventilation, acidosis and hypoglycemia. Aspirin toxicosis can cause hyperthermia in children and cats. There can be an initial hyperpnea in acute aspirin toxicosis due to stimulation of respiratory centers.

### Clinical chemistry and postmortem findings

Metabolic acidosis and increased anion gap have been noted in acute aspirin toxicosis (Roder, 2004a). Other changes are similar to those described with other NSAIDs. Bone marrow suppression can cause anemia in cats (Kore, 1990; McKellar *et al.*, 1991). Lesions similar to other NSAIDs are expected on necropsy. Gastric mucosal lesions have been documented in the pyloric antrum, body and cardiac region of the stomach of dogs (Boulay *et al.*, 1986).

### Management

Diagnosis and treatment of salicylate toxicosis is similar to that described above for NSAIDs. Urine and plasma testing is available at many veterinary and hospital laboratories but there is a poor correlation between plasma salicylate levels and with clinical signs (Talcott, 2006). The presence of salicylate in horse urine is expected, even in horses not given aspirin, and is therefore not a significant finding (Boothe, 2001).

Salicylates can inhibit gastric emptying and gastrointestinal absorption is prolonged with enteric-coated tablets, therefore, gastric lavage is sometimes useful up to 12 hours after exposure. Activated charcoal is most useful within 2 hours of aspirin ingestion. Cathartic use does not appear to decrease absorption.

Forced alkaline diuresis is more useful for aspirin than for other NSAIDs, and is frequently used by physicians. Low urinary pH promotes salicylate elimination by trapping the ionic form in the urine. Alkaline peritoneal dialysis has been recommended for large overdoses (Davis, 1980; Kore, 1990). Acetazolamide, a carbonic anhydrase inhibitor, can be used to alkalinize urine but will exacerbate metabolic acidosis. Acid-base status must be closely monitored in any animal undergoing alkaline diuresis. Monitoring and supportive care for changes in hydration, electrolyte balance and body temperature are warranted. Clearly, use of NSAIDs to treat hyperthermia must be avoided. Diazepam can be used for seizure management.

## COLD, COUGH AND ALLERGY MEDICATIONS

Medications for the symptomatic relief of cold, flu and allergies are common and can contain multiple active ingredients, including analgesics, decongestants, antihistamines, antitussives and expectorants. Other possible ingredients are ethanol and caffeine. These medications can come in a variety of forms, including oral tablets, extended-release tablets, dissolving granules/tablets and as syrup (liquid).

Dextromethorphan is a centrally active antitussive. Structurally similar to opioids, it acts on receptors in medullary cough centers and directly suppresses its activity. Due to its dissociative effects at high doses, increasing illicit use of dextromethorphan as a recreational drug has been reported (Romanelli, 2009). This effect is similar to those of ketamine and phencyclidine, which are both controlled substances discussed in the next chapter. However, dextromethorphan toxicoses are rare in small animals, sedation being the most common effect, and respiratory suppression is unlikely. If an

animal is overdosed with dextromethorphan, activated charcoal can be given early. Treatment consists of observation and supportive and symptomatic care as needed.

Guaifenesin, or glyceryl guaiacolate, is the most common expectorant or mucolytic agent. It is used as a sedative and muscle relaxant at high parental doses in veterinary medicine as an adjunct to anesthesia and for controlling seizures. Mild decrease in blood pressure, increase in cardiac rate and thrombophlebitis are possible side effects of parental exposures (Plumb, 2005). However, it is present in relatively small concentrations in over-the-counter products, and overexposure through oral route will most likely cause only minimal effects such as vomiting and gastritis. Treatment for guaifenesin overdose would include early routine detoxification (emesis is likely to be contraindicated), and symptomatic and supportive care (Papich, 1990).

### Decongestants

Decongestants can be largely divided into sympathomimetic amines such as pseudoephedrine, ephedrine, phenylephrine, phenylpropanolamine and imidazolines such as oxymetazoline, xylometazoline and tetrahydrozoline. Sympathomimetic amines and imidazolines are used as decongestants because of their vasoconstriction effects.

Among these, pseudoephedrine is the most common decongestant associated with toxicosis in small animals, more commonly dogs than cats (Papich, 1990). Pseudoephedrine has been commonly used in cold and allergy preparations, but due to its illicit use in the manufacture of methamphetamine, many U.S. states now regulate its sale. Pseudoephedrine is a stereoisomer of the plant alkaloid ephedrine. Ephedrine is found in *Ephedra* spp. and *Sida cordifolia*. *Ephedra* sp. is used to produce ma huang, an herbal drug used in asthma, allergy and cold formulations, diet pills and in various supplements (Means, 1999, 2005; Ooms and Khan, 2001). Phenylephrine is found in nasal sprays and hemorrhoid creams.

Phenylpropanolamine (PPA) was commonly used in diet pills, cold and allergy products, both as OTC and prescription drugs for humans, and to treat urinary incontinence in dogs (Papich, 1990; Means, 2005). FDA issued a public health advisory in November 2000 due to increased risk of hemorrhagic stroke associated with the use of PPA in humans (Cantu, 2003). Following the advisory, it was voluntarily withdrawn from the market for human use in the United States. Although cardiotoxic effects have been reported in dogs by accidental ingestion (Crandell and Ware, 2005), PPA is still available as a veterinary prescription drug for the control of urinary incontinence in dogs.

Imidazolines are commonly used in over-the-counter eye drops and nasal sprays. Oxymetazoline is found in nasal sprays as a topical decongestant as well as treatment for allergic rhinitis, epistaxis and eye drops (marketed as Visine LR®).

Sympathomimetic amines can interact with digoxin, MAO inhibitors, halothane and methylxanthines (Means, 1999, 2005; Ooms and Khan, 2001). Certain conditions are known to predispose animals to adverse reactions when given sympathomimetic amines. These include diabetes, hypothyroidism, hyperthyroidism, cardiac disease, hypertension, seizure disorders, renal disease and glaucoma.

### Toxicity

Pseudoephedrine is used to improve urethral sphincter tone as a treatment for urinary incontinence in dogs, and is given at a dose of 1 to 2mg every 12 hours (Means, 2005). Clinical toxicosis has been reported in dogs given 5 to 6mg/kg, and death was documented in dogs given 10 to 12mg/kg (Means, 1999, 2005; Ooms and Khan, 2001).

The therapeutic dose of phenylpropanolamine for urinary incontinence in dogs is 1.1mg/kg (Crandell and Ware, 2005; Means, 2005). Elevated blood pressure was seen in beagles given 3.1mg/kg PO every 8 hours, and myocardial damage was noted in a dog given 48mg/kg.

Drugs containing ephedrine, in the form of ma huang, are often combined with caffeine, in the form of guarana, and this combination of drugs acts synergistically, enhancing the toxicity of this product. Doses of 1.3 to 88.9mg/kg ma huang given concurrently with 4.4 to 296.2mg/kg guarana have been associated with clinical toxicosis in dogs. One dog given a dose of 5.8mg/kg ma huang and 19.1mg/kg guarana died. There seems to be great individual difference in sensitivity to this combination of drugs, and prognosis is also dependent on the time that elapses between exposure and treatment (Ooms and Khan, 2001).

### Pharmacokinetics

Decongestants are rapidly absorbed by the gastrointestinal tract (Papich, 1990; Means, 2005). Ephedrine is absorbed within 2 hours of ingestion (Ooms and Khan, 2001). The onset of action is usually within 30 minutes, though it is delayed up to 8 hours with extended release products. Sympathomimetic amines are believed to cross the blood-brain barrier and the placenta, and are known to be secreted into milk. Metabolism occurs primarily in the liver. Most of a dose of ephedrine is excreted unchanged in the urine, and other decongestants are excreted 55 to 75% as the parent compound. The elimination half-life of pseudoephedrine is 2 to 21 hours and that for phenylpropanolamine is 2 to 4 hours. Urinary excretion is accelerated with low urine pH (Ooms and Khan, 2001; Means, 2005).

### Mechanism of action

Adrenergic stimulation is responsible for the effects produced by decongestants (Papich, 1990; Means, 1999, 2005; Ooms and Khan, 2001; Crandell and Ware, 2005). Stimulation of  $\alpha$ -1 receptors causes vasoconstriction and subsequent drying of the mucous membranes of the nasal mucosa and sinuses. Ophthalmic solutions decrease eye redness by constriction of conjunctival and scleral vasculature. Peripheral vasoconstriction leads to increased systemic vascular resistance and hypertension.  $\alpha$ -1 receptor stimulation can cause vasospasm of the coronary artery and myocardial necrosis. Other  $\alpha$ -adrenergic effects may include appetite suppression, CNS stimulation and mydriasis. The effects of phenylephrine and oxymetazoline are  $\alpha$ -receptor specific. Stimulation of  $\alpha$ -receptors by phenylpropanolamine causes release of endogenous catecholamines in the brain and heart, and can inhibit MAO at high doses. Stimulation of  $\beta$ -receptors is the cause of some of the cardiac effects attributed to decongestants, which include increased contractility and output, increased heart rate and tachyarrhythmia. Reflex bradycardia is possible. Bronchodilation is also mediated through  $\beta$ -receptors.

### Clinical signs

CNS stimulation is the common presentation for animals with decongestant overdose. Hyperactivity, restlessness, agitation, pacing and vocalization are seen (Papich, 1990; Means, 1999, 2005). Hallucinatory behaviors in dogs include staring into a corner or at unseen objects, perhaps even biting at them. Tremors, seizures and head-bobbing are observed. Hyperthermia can be secondary to increased activity, and DIC or rhabdomyolysis with associated renal failure are possible outcomes. Cardiovascular changes include tachycardia, reflex bradycardia and hypertension. Hypertension was described in beagles 30 to 60 minutes after dosing. Blood pressure in some dogs remained elevated for 6 hours. Animals can die from cardiovascular collapse. Vomiting, diarrhea, dehydration and anorexia were reported in a dog with phenylpropanolamine toxicosis. This dog was also ataxic, lethargic, tachycardiac and tachypneic. Eyes were bilaterally dilated with vertical nystagmus and loss of pupillary light reflex. This dog was not hypertensive, but it did not present to the veterinarian until 12 hours after ingestion (Crandell and Ware, 2005).

Ooms and Khan (2001) studied dogs accidentally overdosed with combination of ma huang and guarana herbal preparations and found that the onset of clinical signs could be as early as 30 minutes after ingestion, but was usually within 8 hours. Duration of signs ranged from 10 to 48 hours. Vomiting was seen in 47% of dogs, 5% were anorexic, 30% were tachycardic, 6% were tachypneic, 21% had mydriasis, 27% had tremors, 6% had



behavioral changes such as snapping, pacing and head shaking, 6% had seizures, 5% had depression, 5% had weakness and 5% were apprehensive. Hyperthermia was reported in 28% of these dogs.

### *Clinical chemistry*

Hypokalemia, hyperglycemia and hyperinsulinemia are usually reported in dogs with decongestant toxicosis (Means, 1999, 2005). Changes reported in a dog that had ingested phenylpropanolamine included hypoglycemia, mild hyperbilirubemia, and elevated ALT, ALP, BUN, creatinine, and CK. Blood pH and calcium levels were elevated. Polycythemia and thrombocytopenia were noted. A urine sample contained blood, hemoglobin or myoglobin, and protein. Elevated serum troponin indicates myocardial damage (Crandell and Ware, 2005).

### *Diagnosis and management*

Diagnosis of decongestant toxicosis is usually based on history and clinical signs. Some laboratories are able to test for pseudoephedrine, ephedrine and phenylpropanolamine in plasma or urine to confirm exposure, but it takes hours or days for results to be available, thus treatment must be initiated in the poisoning case before analytical results become available.

Treatment consists of detoxification, symptomatic and supportive care. Emetics use is risky due to the potential for aspiration. Gastric lavage can be performed in the stabilized, anesthetized and intubated patient after a large ingestion. Activated charcoal and cathartic should be given. Blood pressure, ECG and body temperature should be monitored closely. CBC, serum chemistry and acid-base status should be monitored every one to three days.

Tachycardia is treated with  $\beta$ -blockers. Propranolol can be administered at a dose of 0.02 to 0.06 mg/kg slowly by IV (Means, 1999, 2005; Ooms and Khan, 2001). Propranolol therapy helps to stabilize hypokalemia. Alternately, lidocaine has been used at a dose of 2 mg/kg IV by intermittent bolus or by continuous infusion at a rate of 80  $\mu$ g/kg per minute (Crandell and Ware, 2005). Crandell and Ware (2005) recommended atenolol at 0.2 mg/kg every 12 hours and enalapril 0.5 mg/kg every 12 hours, both given orally, to support myocardial function for phenylpropanolamine toxicosis. Papich (1990) recommended atropine at a dose of 0.04 mg/kg subcutaneously (SC) or IV. High peripheral vascular resistance and hypertension are relieved with  $\alpha$ -adrenergic receptor blocking agents. Prazosin can be given at 1 to 2 mg every 8 hours or phentolamine can be given at 0.1 mg/kg IV as needed (Papich, 1990; Ooms and Khan, 2001).

Severe CNS stimulation sometimes requires treatment with more than one anticonvulsant. Though acepromazine and chlorpromazine have historically been

believed to decrease the seizure threshold, they can be used to treat pseudoephedrine or ephedrine toxicosis, which induce seizures by a different mechanism. Acepromazine is given at a dose of 0.05 to 1.0 mg/kg IM, IV or SC, starting with a low dose and adding more as needed. Alternately, chlorpromazine is given IM or IV at a dose of 0.5 to 1.0 mg/kg, starting with a low dose and increasing as needed. If needed, phenobarbital can be given for refractory seizures at a starting dose of 3 mg/kg to effect (Means, 1999, 2005). Isoflurane anesthesia can be used for severe clinical signs (Ooms and Khan, 2001). Use of diazepam or other benzodiazepines is considered contraindicated because the dissociative effects of this drug class can exacerbate clinical signs of sympathomimetic amines.

Fluid therapy and cautious urinary acidification can help promote excretion (Ooms and Khan, 2001; Means, 2005). Urinary acidifiers enhance excretion of pseudoephedrine in humans and may be used in dogs. Acid-base status must be monitored closely when acidifiers are used. The dose for ascorbic acid is 20 to 30 mg/kg IM or IV every 8 hours. Ammonium chloride is given 50 mg/kg PO every 6 hours. Glucose is added to intravenous fluids to treat hypoglycemia. Overhydration must be avoided to prevent pulmonary edema in the hypertensive patient.

Adverse clinical signs associated with decongestants can last for 72 hours or more (Means, 2005). One dog with severe clinical signs after phenylpropanolamine ingestion recovered after six days of hospitalization (Crandell and Ware, 2005). Most animals respond to treatment more rapidly. Clinical signs that have been associated with an unfavorable outcome include uncontrollable seizures, DIC, myoglobinuria and head-bobbing. Ooms and Khan (2001) report that 26 out of 34 dogs recovered, usually within 10 to 48 hours, with treatment after ingestion of a ma huang and guarana combination drug. The remaining eight dogs died or were euthanized.

### **Antihistamines**

Antihistamines act by competitive inhibition of histamine at histamine receptors. Compounds referred to as antihistamines in this section are the  $H_1$  histamine receptor antagonists.  $H_2$  histamine receptor antagonists are also sold over the counter and are covered later, with drugs affecting the gastrointestinal system. They will be referred to as  $H_2$  histamine receptor antagonists (or  $H_2$ -blockers).

Many antihistamines, including brompheniramine, chlorpheniramine, clemastine, diphenhydramine, loratadine and triprolidine, are found in allergy, cold and flu formulations. Others, such as dimenhydrinate and meclizine, are used as antiemetics to treat motion sickness. Doxylamine



is used as a sedative in sleep aids. Hydroxyzine is used by veterinarians to treat canine atopy (Tegzes *et al.*, 2002).

### Toxicity

An incomplete list of antihistamines is provided in Table 28.3. Chlorpheniramine is the antihistamine most commonly associated with adverse effects in dogs (Papich, 1990; Gwaltney-Brant, 2004). The oral LD<sub>50</sub> for clemastine in dogs is 175 mg/kg. The therapeutic dose for hydroxyzine is 2.2 mg/kg, and 111 mg/kg is reported as toxic in dogs (Tegzes *et al.*, 2002). Terfenadine, which is no longer sold in the U.S., caused clinical signs of toxicosis in a dog at a dose of 6.6 mg/kg, but there is considerable individual difference in sensitivity to this drug (Otto and Greentree, 1994; Gwaltney-Brant, 2004). Electroencephalogram changes were noted in dogs dosed with 30 mg/kg, clinical signs were noted in dogs given in 100 mg terfenadine/kg per day for 2 to 3 weeks,

and vomiting was a consistent finding in dogs dosed with 150 mg/kg.

### Pharmacokinetics

Antihistamines are well absorbed by the monogastric gastrointestinal tract, but oral doses are poorly absorbed in ruminants (Adams, 2001; Gwaltney-Brant, 2004). The anticholinergic effects of antihistamines can slow absorption by delaying gastric emptying. Peak plasma concentrations usually occur within 2 to 4 hours of ingestion and the onset of clinical effects tends to be 20 to 45 minutes after ingestion. Therapeutic effects usually last from 3 to 12 hours. Antihistamines are highly protein bound. First generation antihistamines are able to freely cross the blood–brain barrier and are more likely to cause CNS effects than second generation products, which do not normally enter the CNS. Second generation antihistamines cross the blood–brain barrier if given at very high doses. Terfenadine has been detected in cerebrospinal fluid in overdose situations (Otto and Greentree, 1994).

Metabolism of antihistamines takes place predominantly in the liver. Hydroxyzine is metabolized to the active product cetirizine, which does not cross the blood–brain barrier. The elimination half-life of antihistamines is dependent on the individual compound. Most metabolites are excreted in the urine, though there is some biliary excretion of terfenadine. Antihistamines excreted into the bile can undergo enterohepatic cycling.

TABLE 28.3 Antihistamines (H<sub>1</sub> histamine receptor inhibitors). See Gwaltney-Brant (2006) for more detailed information

First generation	Second generation
<b>Alkylamines</b>	<b>Piperadines</b>
Brompheniramine	Terfenadine
Chlorpheniramine	Astemizole
Dexbrompheniramine	Levocabastine
Dexchlorpheniramine	Loratadine
Dimetindene	
Pheniramine	<b>Third generation</b>
Triprolidine	Desloratadine
<b>Ethanolamines</b>	Fexofenadine
Clemastine	Levocetirizine
Dimenhydrinate	
Diphenhydramine	
Bromodiphenhydramine	
Carbinoxamine	
Doxylamine	
Phenyltoloxamine	
<b>Ethylenediamines</b>	
Antazoline	
Pyrilamine	
Tripelennamine	
<b>Phenothiazines</b>	
Methdilazine	
Trimeprazine	
<b>Piperazines</b>	
Hydroxyzine	
Cyproheptadine	
Meclozine	
Cyclizine	
Bucizine	
Chlorcyclizine	
Niaprazine	
Certirizine	
<b>Tricyclics</b>	
Promethazine	

### Mechanism of action

Antihistamines act by competitive inhibition of histamine at H<sub>1</sub> receptors (Papich, 1990; Gwaltney-Brant, 2004). Binding is reversible, but can become irreversible or slow to dissociate at high doses, as with terfenadine (Otto and Greentree, 1994; Gwaltney-Brant, 2004). H<sub>1</sub> receptors are found in a variety of tissues including: mast cells of the skin; smooth muscle of airways, gastrointestinal tract, urogenital tract and cardiovascular system; endothelial cells and lymphocytes; and mammalian CNS. Histamine produces dermal itching and allergic responses in the skin and contraction of smooth muscle in the bronchial tree and intestine. Low doses of histamine produce a rapid onset of vascular dilation. The wheal-and-flair reaction associated with histamine release is due to increased vascular permeability. Histamine in the CNS modulates sleep/wake cycles.

Antihistamines are used to block allergic response and reduce itching. Antihistamines block smooth muscle contraction, reducing bronchoconstriction and affecting vascular and uterine smooth muscle. Effects on intestinal smooth muscle can cause gastrointestinal disturbances. Antihistamines also prevent increases in vascular permeability associated with histamine release. CNS effects produced by antihistamines can include sedation or excitement.

Muscarinic stimulation is believed to be involved in motion sickness-induced vomiting. The antimuscarinic actions of antihistamines decrease nausea and vomiting (Papich, 1990). Gastrointestinal motility is decreased, and respiratory suppression is sometimes seen with antihistamines. Phenothiazine-type antihistamines also block  $\alpha$ -adrenergic receptors. Allergic reactions to antihistamines have been noted.

### Clinical signs

Clinical signs of antihistamine overdose are usually evident within half an hour of dosing. Signs of CNS depression often occur with therapeutic doses of first-generation antihistamines and include sedation, ataxia and drowsiness. More severe clinical signs such as profound depression, coma and respiratory suppression incompatible with life can occur. A dog with hydroxyzine toxicosis presented with tachycardia and weakness progressing to stupor, coma, loss of gag reflex and apnea (Tegzes *et al.*, 2002). Higher doses of antihistamines can have a stimulatory effect on the CNS, particularly in children and young animals. These effects are less common in adults. Overdosed individuals appear to experience hallucinations, lack of coordination, disorientation, irritability, anxiety, aggression, seizures and pyrexia.

Vomiting and diarrhea have been associated with first-generation antihistamines. Anticholinergic effects include dry mucous membranes, fixed and dilated pupils, tachycardia and arrhythmia, and animals can be either hypertensive or hypotensive. Cardiac abnormalities were documented in humans and dogs that ingested terfenadine (Otto and Greentree, 1994; Gwaltney-Brant, 2004). Animals can have allergic reactions to topical or oral antihistamines. A list of associated clinical signs includes dermatitis, pyrexia and photosensitization (Gwaltney-Brant, 2004). Teratogenic effects have been detected in experimental animals treated with piperazine.

Metabolic acidosis and electrolyte abnormalities have been documented based on serum chemistry of antihistamine overdosed animals, but overall changes are non-specific. Changes reported on postmortem examination also tend to be nonspecific. Rhabdomyolysis and associated renal lesions or DIC are possible complications of antihistamine toxicosis.

### Management

Diagnosis of antihistamine toxicosis is usually based on history and clinical signs. Laboratory testing of urine or plasma can be helpful to confirm exposure, but results will be delayed for hours or days and quantitation is unlikely to be of value.

Emetics are appropriate to promote gastric emptying in asymptomatic animals that recently ingested large doses of antihistamines, but caution is advised because

onset of clinical signs is sometimes rapid. Gastric lavage of the anesthetized, intubated animal is more appropriate in the treatment of symptomatic animals. Activated charcoal and a cathartic are instilled after lavage or given to the stable patient. Multiple doses of activated charcoal are needed to interrupt enterohepatic cycling.

Drugs such as penicillin G and NSAIDs have been recommended to reduce protein binding and enhance excretion (Tegzes *et al.*, 2002). However, this may worsen clinical signs in the short term by making the antihistamine more bioavailable.

Serum chemistry should be assessed and monitored for hydration, electrolyte balance, acid-base status, and liver and kidney function. Animals with poor hepatic or renal function can have a decreased rate of antihistamine elimination (Gwaltney-Brant, 2004). Cardiac function, blood pressure and body temperature should be monitored as well. Respiratory function should be closely monitored and intubation is sometimes required to support the comatose patient.

Fluid therapy is useful to maintain hydration and for cardiac support and diuresis. Imbalances in electrolytes and pH need to be corrected. Animals rarely require treatment for hypotension or mild to moderate cardiac arrhythmias, which will usually respond to fluid therapy. Epinephrine should not be used to treat promethazine overdose (Staley and Staley, 1995). Promethazine inhibits adrenergic receptors and addition of epinephrine can lead to further decrease in blood pressure. Atropine should not be used, as it can potentiate the anticholinergic effects of antihistamines.

Seizures can be treated cautiously with benzodiazepines or short-acting barbiturates. The depressive effects of antihistamines will be additive with the effects of sedatives, causing "rebound depression." Though cholinergic signs are sometimes present, treatment with physostigmine is contraindicated as it potentiates cardiovascular dysfunction and seizures.

Animals overdosed with antihistamines usually improve within 24 hours, though signs sometimes persist for up to three days. Prognosis is dependent on the severity of signs and is guarded in animals presenting with seizures or coma (Gwaltney-Brant, 2004). Tegzes *et al.* (2002) report recovery of a comatose dog that had been overdosed with hydroxyzine, though the authors note that a week or two of supportive care could be required in such cases.

## DRUGS USED TO TREAT GASTROINTESTINAL SYMPTOMS

This classification includes antacids, laxatives and anti-diarrheal drugs.

## Antacids

H<sub>2</sub> histamine receptor antagonists and mineral antacids are used to increase gastric pH and associated discomfort. Use of these products to aide in healing of gastroduodenal ulcers secondary to NSAID use is noted in a previous section. Few adverse effects have been reported with these drugs.

### H<sub>2</sub> histamine receptor antagonists

H<sub>2</sub> histamine receptor antagonists include cimetidine, famotidine, nizatidine and ranitidine. Cimetidine impairs gastrointestinal absorption of some drugs and prolongs the effects of others by inhibiting their metabolism by microsomal enzymes.

#### Pharmacokinetics

Cimetidine is rapidly absorbed and 70% of a given dose is bioavailable. Absorption can be slowed by the presence of food in the stomach. The mean absorption time for ranitidine was approximately an hour and absorption was not impaired by the presence of food in the stomach. Ranitidine is 73% bioavailable in dogs and 27% available in horses after oral dosing. Famotidine is poorly absorbed and only 37% bioavailable. Nizatidine is rapidly and almost completely absorbed (Boothe, 2001). H<sub>2</sub> receptor antagonists (or H<sub>2</sub>-blockers) are less lipid soluble than H<sub>1</sub> receptor antagonists (or H<sub>1</sub>-blockers) and are less likely to cross the blood-brain barrier. Ranitidine is about 15% protein bound.

Cimetidine and ranitidine are metabolized predominantly in the liver. Seventy-three percent of an oral dose of ranitidine undergoes hepatic metabolism in people, compared to 40% in dogs. Unchanged cimetidine, famotidine and nizatidine are excreted in the urine. The plasma half-life for cimetidine is about an hour, but can be prolonged in individuals with renal or hepatic insufficiency. The elimination half-life of ranitidine is 2.5 hours in humans and 4 hours in beagles (Boothe, 2001).

#### Mechanism of action

H<sub>2</sub> receptor antagonists specifically bind to and block the actions of H<sub>2</sub> receptors by competitive inhibition. H<sub>2</sub> receptors are present in the enterochromaffin cells of the gastric mucosa and histamine stimulates acid secretion and, to a limited extent, pepsin secretion (Adams, 2001; Tegzes *et al.*, 2002). High doses of histamine produce a slow onset, prolonged vasodilatory response. H<sub>2</sub> receptor inhibitors decrease acid and pepsin secretion. They also block some of the cardiovascular effects of histamine (Adams, 2001). Famotidine is nine times more potent as a H<sub>2</sub>-blocker than ranitidine, which is five to 12 times more potent than cimetidine. Famotidine also has the longest duration of clinical effects (Boothe, 2001).

Cimetidine reduces hepatic blood flow by around 20% and inhibits microsomal cytochrome P450 enzymes, thus it interferes with metabolism of other drugs.

#### Clinical signs

Minor side effects can be expected to occur in animals.

### Mineral antacids

A list of common active ingredients in mineral antacids can be found in Table 28.4. Products may contain more than one active ingredient. Sodium bicarbonate is present in baking soda and in effervescent antacid products. These products are common in many households, and may be accidentally ingested by pets or administered by pet owners. They are used in veterinary medicine to decrease gastric hyperacidity and treat peptic ulcers, uremic ulcers, reflux esophagitis and rumen acidosis secondary to grain overload (Boothe, 2001). Few adverse effects are associated with mineral antacids (Papich, 1990).

Sodium bicarbonate and calcium carbonate are absorbed after ingestion. Transcutaneous absorption of sodium bicarbonate across damaged skin can occur (Gonzalez and Hogg, 1981). Some of the magnesium in magnesium salt-containing antacids is absorbed, and hypermagnesemia has been reported with repeated dosing (Boothe, 2001).

Mineral antacids increase gastric pH. Rebound acid secretion may occur when dosing is discontinued. Some mineral antacids inactivate pepsin and some bind bile salts (Boothe, 2001). Magnesium-containing salts have a laxative effect, and aluminum-containing salts have a constipating effect, thus these active ingredients are often used in combination. Aluminum complexes phosphate in the intestine and aluminum hydroxide has been used to decrease phosphate absorption in patients with renal disease (Segev *et al.*, 2008).

Carbon dioxide is produced rapidly when sodium bicarbonate is introduced to an acid, and this reaction may cause gastric distension (Boothe, 2001). Chronic use of calcium carbonate antacids may produce metabolic

TABLE 28.4 An incomplete list of mineral antacids

<b>Aluminum salts</b>
Aluminum hydroxide
Aluminum magnesium silicate
Aluminum phosphate
<b>Magnesium salts</b>
Magnesium hydroxide
Magnesium oxide
Magnesium silicate
<b>Others</b>
Calcium carbonate
Sodium bicarbonate

acidosis, hypercalcemia, hypophosphatemia and calciuria with urolithiasis and metastatic calcification (Fitzgibbons and Snoey, 1999; Boothe, 2001).

Laxatives, cathartics and enemas

Drugs in this category are designated as bulk-forming laxatives, lubricant laxatives, irritant laxatives, hyperosmotic cathartics or enemas.

Bulk-forming laxatives

Nonabsorbable polysaccharide cellulose derivatives are used as bulk-forming laxatives. Natural sources include psyllium or plantago seed, wheat bran and fruits such as prunes. Synthetic forms include methylcellulose and carboxymethylcellulose. These products act by absorbing water in the gastrointestinal tract and swelling the softened fecal mass, resulting in intestinal distension and reflex peristaltic contractions. Intestinal bacteria act on cellulose and hemicellulose and produce volatile fatty acids, enhancing the osmotic effect. Fluid feces and tympany can result (Boothe, 2001). No serious adverse effects have been reported in companion animals, though these products cause fluid and electrolyte loss secondary to diarrhea. Papich (1990) suggests that subsequent dehydration can lead to intestinal impaction. Animals treated with bulk-forming laxatives should have fresh water available. If diarrhea occurs, monitor hydration and electrolyte status and correct as needed.

Lubricant laxatives

Lubricant laxatives, including mineral oil and white petrolatum, are hydrocarbon mixtures derived from petroleum. Mineral oil is frequently used in large animals and white petrolatum products are used to treat trichobezoars in cats (Papich, 1990). These large hydrocarbons are minimally absorbed and act by coating feces with a film that entraps moisture and lubricates passage. Hydrocarbon laxatives reduce absorption of fat soluble vitamins and possibly other nutrients, thus chronic use can produce deficiencies. The small amount of absorbed hydrocarbons can provide a nidus for granuloma formation in the intestinal mucosa, mesenteric lymph nodes or liver. Adverse effects, however, are rarely reported with lubricant laxatives.

Irritant laxatives

Table 28.5 lists common types of irritant laxative. Phenolphthalein laxatives are often chocolate flavored, increasing palatability, but are only effective in primates and swine (Boothe, 2001). Bisacodyl is similar to phenolphthalein. Vegetable oil products contain irritant fatty

TABLE 28.5 Irritant cathartics

<b>Diphenylmethanes</b>
Phenolphthalein
Bisacodyl
<b>Vegetable oils</b>
Castor oil
Raw linseed oil
Olive oil
<b>Anthraquinones</b>
Cascara sagrada
Senna
Aloin

acids such as ricinoleic acid in castor oil, linoleates in linseed oil and oliveates in olive oil. Common anthraquinone-type cathartics are derived from plants: cascara sagrada from *Rhamnus* spp., senna from *Senna* spp. and aloin from *Aloe* spp. Danthron (1,8-dihydroxyanthroquinone) is synthetic, but is considered the prototype for the anthraquinone laxative group.

Five percent of an oral dose of bisacodyl is absorbed. There is some absorption of anthraquinone glycosides, and delayed transit through the small intestine decreases effectiveness. Anthraquinones are secreted in the milk, causing clinical effects in the nursing young (Boothe, 2001).

As suggested by the term irritant laxative, these compounds cause contact irritation of gastrointestinal mucosa and increase fluid secretion into the lumen. Diarrhea occurs 6 to 8 hours after diphenylmethane cathartics are administered and can be severe if large doses are given (Boothe, 2001). Pink discoloration of alkaline urine is seen with phenolphthalein. Acidic urine will turn pink with addition of sodium hydroxide or sodium bicarbonate (Papich, 1990).

Vegetable oil laxatives are hydrolyzed by lipase in the small intestine and form sodium and potassium salts which act as soaps, producing irritation (Boothe, 2001). Ricinoleic acid is the most potent of these compounds, and initiates rapid and complete colonic emptying. This clinical effect is seen 4 to 8 hours after administration of castor oil in small animals, and 12 to 18 hours post-dosing in large animals. Animals treated with castor oil should be fed moist, bulky material afterward.

Anthraquinones are hydrolyzed by bacteria in the large intestine to emodins which stimulate the myenteric plexus. Anthraquinones produce catharsis after 6 to 12 hours in small animals and 12 to 36 hours in large animals (Boothe, 2001). Catharsis is accompanied by reduced hydration and electrolyte loss. Abdominal pain or colic is produced by large doses. Changes in urine color are reported with anthraquinones. With chronic use of these laxatives, the myenteric plexus will degenerate causing a loss in intestinal motility. Animals treated with irritant cathartics should be monitored for hydration and electrolyte status with correction as needed.



Some irritant cathartics increase prostaglandin synthesis, and NSAIDs minimize this effect.

### *Hyperosmotic cathartics*

Magnesium sulfate, or Epsom salt, is a common osmotic cathartic and is used in 6% isotonic solution. Other magnesium-containing cathartics include magnesium hydroxide (milk of magnesia), magnesium oxide and magnesium citrate. Sodium sulfate (Glauber's salt), sodium phosphate, potassium sodium tartrate and sodium tartrate (Rochelle salt) are used as cathartics and ingestion of large quantities of sodium chloride produces catharsis. The sugar alcohols mannitol and sorbitol, and synthetic disaccharides such as lactulose, are also used as cathartics. Polyethylene glycol 3350 (PEG) once available only with prescription for whole-bowel irrigation before colonoscopy procedures and to treat body-packer patients (Farmer *et al.*, 2003) is currently available over the counter (MiraLAX®).

Osmotic cathartic use should be avoided in dehydrated animals, and water should be freely available. Other contraindications for use include congestive heart failure, gastrointestinal stasis and hepatic or renal impairment (Henninger and Horst, 1997; Ezri *et al.*, 2006). Hyperosmotic cathartics draw water into the intestinal tract via osmosis. Intestinal distension promotes motility. Effects are usually evident 3 to 12 hours after dosing in monogastrics and within 18 hours in ruminants.

There is minimal slow absorption of most cathartics, but up to 20% of the magnesium in a dose of magnesium sulfate is absorbed in the small intestine (Henninger and Horst, 1997; Boothe, 2001). Renal failure enhances systemic magnesium accumulation, leading eventually to hypermagnesemia. Administration of magnesium sulfate with dioctyl sodium sulfosuccinate in horses increases magnesium absorption due to mucosal damage (Henninger and Horst, 1997). Magnesium ions promote the release of cholecystokinin which promotes peristalsis. Systemic effects of magnesium include inhibition of calcium ion release at neuromuscular junctions, inhibition of acetylcholine release, decreased sensitivity of motor endplates and decreased excitability of myocyte membranes, leading to paralysis. Clinical signs of hypermagnesemia secondary to magnesium sulfate administration in the horse occurred within 6 hours of administration and included perspiration, progressive tremors, recumbence, severe tachycardia, tachypnea, pale mucous membranes, prolonged capillary refill times, flaccid paralysis of the head and neck, and loss of flexor and perineal reflexes. Horses remained alert.

Absorbed phosphate from phosphate containing cathartics can deplete intracellular potassium ions and induce hypokalemia. Lactulose reaches the colon largely intact and is degraded into lactic acid and acetic acid by

large intestinal microflora. Gas is generated and can cause tympany. The associated decrease in pH has been used for ion trapping of ammonium to prevent hepatic encephalopathy in animals with liver failure (Boothe, 2001).

Dehydration and electrolyte imbalances are the most common changes associated with cathartics (Papich, 1990). Management is aimed at correcting these imbalances. Calcium is used to treat hypermagnesemia. Calcium ions displace magnesium ions from cell membranes. Response to therapy is rapid, but repeated dosing with calcium gluconate is sometimes needed. Diuresis with IV fluids and furosemide promote renal excretion. Henninger and Horst (1997) reported that horses treated for hypermagnesemia had serum magnesium concentrations within reference ranges the next day. Electrolyte changes in asymptomatic patients given oral sodium phosphate for pre-surgical catharsis returned to normal within 24 hours (Ezri *et al.*, 2006).

### *Enemas*

An enema is a material given intrarectally to induce defecation. Commonly used enemas include soft anionic soap in water, isotonic or hypertonic sodium chloride, sorbitol, glycerol, sodium lauryl sulfate, sulfoacetate, mineral oil, olive oil and phosphate salts (Boothe, 2001).

Toxicosis has been reported in people and small animals administered hypertonic sodium phosphate solutions, or fleet enemas. Debilitated cats are commonly affected (Papich, 1990). Predisposing factors include administration of a full-strength fleet enema (intended for human use) to a small animal, dehydration, preexisting electrolyte abnormalities, renal or hepatic dysfunction, colonic dilatation and colonic ulceration. A 60 mL hypertonic phosphate enema can cause toxicosis in a cat (Roder, 2004b).

Sodium and phosphate are absorbed in the colon, and absorption is increased with disruption of the mucosa or in animals with chronic constipation (Jorgensen *et al.*, 1985). Uptake of phosphate promotes cellular uptake of calcium, and phosphate can directly bind serum calcium, both contributing to hypocalcemia. Adverse effects associated with sodium phosphate enemas usually occur within an hour of dosing. Vomiting and bloody diarrhea have been observed. Nervous signs that have been reported include depression, ataxia, anxiety, neuromuscular irritability and convulsions. Dehydration, weak pulses, tachycardia, hypothermia, shock and death have also been observed. Serum chemistry in these patients often reveals hyperphosphatemia, hypernatremia, hypocalcemia, metabolic acidosis and hyperglycemia. Hyperkalemia or hypokalemia can occur.

Intensive fluid therapy is required in acute sodium phosphate enema overdose situations. The purpose is to correct dehydration, electrolyte imbalances, acid-base

imbalances, hypoglycemia and treat circulatory shock. Isotonic saline solution can increase renal calcium loss and thus should be avoided. Saline solutions containing 0.45% sodium chloride and 2.5% dextrose, or 2.5 to 5.0% dextrose solutions, have been recommended for hypoglycemic patients. Lactated Ringers solution can be used in hyperglycemic patients. Fluid therapy may be needed for four days. Jorgensen *et al.* (1985) suggested instillation of aluminum carbonate or aluminum hydroxide to bind phosphate in the intestine and maintain body temperature. Prophylactic antibiotics are given for compromise of the colonic mucosa. Intoxicated cats showed improvement within 6 h of initiation of therapy, and electrolyte imbalances resolved within two days.

### Antidiarrheal drugs

Active ingredients in products used to treat diarrhea include bismuth subsalicylate, kaolin-pectin and opioid receptor agonists. Most of the toxic effects associated with bismuth subsalicylate are due to the salicylate component; therefore this formulation is discussed in more detail with aspirin and salicylates. Bismuth is believed to adsorb bacterial endotoxin and have a direct antimicrobial effect.

Kaolin and pectin were previously the active ingredients in the popular over-the-counter product Kaopectate, but bismuth subsalicylate is now used. Kaolin is hydrated aluminum silicate and acts as an adsorbent. Pectin is a carbohydrate extracted from citrus fruit that acts as an adsorbed and intestinal protectant. Toxicosis attributed to kaolin-pectin preparations has not been observed, though these products are likely to decrease absorption of other drugs.

The over-the-counter drug Imodium® and the prescription drug Lomotil® contain the opioid receptor agonists loperamide and diphenoxylate, respectively. Loperamide is sold in 2mg capsules and liquids at a concentration of 0.20mg/mL. The effective dose for loperamide is 0.08mL/kg PO every 12 hours in small dogs and cats. These products are poorly absorbed. They function to decrease gastrointestinal secretion and motility. Side effects include constipation, abdominal pain, vomiting and drowsiness. Collies and other dog breeds with a mutation in the *ABCB1* gene (formerly *MDR1*) are believed to have increased susceptibility to loperamide (Sartor *et al.*, 2004). Clinical signs in collies include salivation, weakness, ataxia and disorientation. Management consists of monitoring and supportive and symptomatic care. Activated charcoal can be given to decrease intestinal absorption in the case of a large ingestion. Neuromuscular signs in one collie resolved about a day after the drug was discontinued (Sartor *et al.*, 2004). The opioid antagonist naloxone is used to manage severe clinical signs, but is usually

unnecessary. Naloxone has a short half-life, requiring repeated dosing as needed (Papich, 1990).

### TOPICAL DRUGS

Toxicosis secondary to topical preparations can occur through dermal absorption or ingestion. Cats in particular are fastidious groomers and any product that is used on a cat is likely to end up in the cat. Some animals, dogs in particular, will chew through a tube containing a topical drug (Papich, 1990).

Like other OTC drugs, topical products frequently contain more than one active ingredient. Antibiotic preparations can contain bacitracin, neomycin and polymyxin. Though rare, topical hypersensitivities have been reported, these antimicrobials are poorly absorbed from the normal gastrointestinal tract, limiting their systemic effects (Papich, 1990). However, if large quantities are ingested, damage to the resident gastrointestinal microflora can lead to diarrhea and discomfort. Use of these products in hamsters or guinea pigs, which are predisposed to develop antibiotic-induced enterocolitis, should probably be avoided.

Benzoyl peroxide is present in some topical antiseptics and acne medications. It acts by releasing hydrogen peroxide, which has antibacterial and keratinolytic effects and promotes epithelial cell turnover. Hyperemia and blistering have been reported with overuse of products containing benzoyl peroxide. Ingestion can lead to intra-gastrointestinal gas production and distension. Emetics are contraindicated if this should happen. Gastric decompression requires use of a stomach tube.

### CONCLUSIONS

OTC drugs, which are widely available to consumers, encompass a large number of products, many containing multiple active ingredients, developed for oral, topical, intraocular, intranasal and intrarectal administration. Of the thousands of OTC drugs available, only a fraction were addressed in this chapter, including analgesics, cold, flu and allergy medications, and drugs used to treat gastrointestinal symptoms. Obtaining a thorough history is of great importance when dealing with suspect OTC drug-related problems. It is often helpful if the owner brings the drug container to the veterinarian as a source of information on the active ingredients, indications for use and manufacturer identification, telephone number or address. Treatment for most OTC drugs includes decontamination, stabilization and symptomatic and supportive care with careful monitoring.

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# Toxicity of drugs of abuse

Karyn Bischoff

## INTRODUCTION

Potential for exposure to illegal drugs exists for many companion animals, horses and even livestock on occasion. Forty-seven percent of graduating American high-school students surveyed had used illegal drugs in some form (Latimer and Zur, 2010). The illegal drug trade in the United States peaked in the late 1970s, declined in the 1980s and early 1990s, and increased again through the late 1990s to stabilize since 2000 (Compton *et al.*, 2005).

Marijuana is one of the most prevalent recreational drugs in the world after legal substances ethanol and nicotine (Janczyk *et al.*, 2004; Compton *et al.*, 2005; Johnson *et al.*, 2005; Vitale and van de Mheen, 2005; Latimer and Zur, 2010). Cocaine is probably second in popularity among illegal substances in some areas of the United States and Europe (Kisseberth and Trammel, 1990; Dumonceaux, 1995; Queiroz-Neto *et al.*, 2002; Vitale and van de Mheen, 2005). Since the 1980s, the term “club drugs” has come into use, representing drugs frequently found at nightclubs and all-night “rave” parties. Club drugs are a continuing trend (Smith *et al.*, 2002; Banken, 2004). Many are stimulants, though depressants may be used to counter the effects of the stimulants or given secretly to sedate a victim for the purpose of theft assault. Hallucinogens are also used. Some of the most common club drugs include ketamine, 3,4-methylenedioxymethamphetamine (MDMA), commonly called “ecstasy,” flunitrazepam (Rohypnol®), the “date rape drug,” and  $\gamma$ -hydroxybutanoic acid (GHB). Beginning in late 2010, the abuse of the legal substances mephedrone and methylenedioxypyrovalerone, marketed as “bath salts,” has come into vogue, prompting an emergency order to remove them from the market in Louisiana.

## Illicit drugs and small animals

Among companion animals, dogs are the most susceptible to poisoning with illicit substances, though toxicoses occasionally arise in cats, ferrets, birds or other household pets. Exposure may be through voluntary ingestion or malicious poisoning and other forms of animal abuse (Kesseberth and Trammel, 1990). There are reports of adolescents entertaining themselves by intoxicating dogs, cats and birds with second-hand marijuana smoke (Schwartz and Riddle, 1985; Buchta, 1988).

Police dogs are at particular risk for ingestion of illegal drugs. They may come into contact with large quantities of the high-purity chemicals in the line of duty (Dumonceaux and Beasley, 1990; Kesseberth and Trammel, 1990). They may ingest whole bags of drugs which must be removed surgically or via endoscopy to prevent rupture and massive exposure. Police dogs may be at increased risk for malicious poisonings. Recently, news reports document the use of dogs as “drug mules” to move bags of heroin, which were surgically implanted. Deaths in several of these dogs were attributed to secondary infections.

Illegal drug ingestion in small animals presents a diagnostic challenge, and often an ethical challenge, to the clinician. Pet owners may not be aware of what the animal was exposed to, as in the case of animal intoxication by adolescents (Schwartz and Riddle, 1985; Buchta, 1988). Jones (1978) reported that a dog ingested illicit substances from a neighbor’s garbage can. Violence is intrinsic to the drug culture in poor neighborhoods (Johnson and Myron, 1995). It could take the form of malicious poisoning or other forms of animal abuse. Suspected malicious poisoning with illicit drugs has been reported (Bischoff *et al.*, 1998).

The pet owner who is aware that the animal ingested an illegal substance may be reluctant to admit it (Godbold *et al.*, 1979; Kisseberth and Trammel, 1990; Dumonceaux, 1995; Welshman, 1996; Frazier *et al.*, 1998; Janczyk *et al.*, 2004; Volmer, 2005). A veterinarian must balance client confidentiality with legal obligation. The client bringing the pet to a veterinary clinic is aware that it requires medical attention, but the threat of legal action can discourage them from disclosing critical information. It is prudent for the veterinarian to be aware of the local laws concerning animal abuse, drug possession and their responsibility towards reporting illegal activities. Sadly, animals have been euthanized in part due to an incomplete history (Smith, 1988). The owner must be made aware that a proper history is required if appropriate treatment is to be administered.

Diagnostic laboratories have screens available to detect the presence of many illegal drugs, but these screens take time and may be expensive (Janczyk *et al.*, 2004). New over-the-counter urine testing kits are available at pharmacies. One study has found these kits useful in detection of barbiturates, some opioids, benzodiazepines and amphetamines in canine urine, but less accurate in the detection of other opioids and marijuana (Teitler, 2009). Awareness of the animal's home situation, including the neighborhood or the presence of adolescent children in the household, may be helpful in determining the potential for exposure to illegal substances. The astute clinician might ask if there was a party in the home when the pet became ill (Kesseberth and Trammel, 1990). Pet owners who do admit that their pet ingested illegal drugs may not be fully aware of what was ingested, or know only the street name of the drug. Drug dealers may combine drugs or make substitutions and many drugs are very similar in appearance.

## Illicit drugs and large animals

Horses may be "doped" to improve athletic performance to hide soundness or temperament problems. Cocaine has been detected in urine samples from horses at athletic events (Queiroz-Neto *et al.*, 2002; Kollias-Baker *et al.*, 2003). It is expected that such drugs would be given in relatively small doses, but dose miscalculations can occur. Exposure to plants grown for illicit drug manufacture has occurred in herbivores. There are reports of lethal marijuana exposure in cattle fed the plant as hay and horses on pasture (Cardassis, 1951; Driemeier, 1997).

Anhydrous ammonia is an ingredient in illegal methamphetamine production. It is also used to instill nitrogen into the soil for fertilization and tanks are sometimes stored near animal facilities. Unscrupulous manufacturers of the drug often steal anhydrous ammonia from farm tanks and may fail to close the tank valves before

leaving the premises. The death of 64 out of 260 dairy cattle in Michigan resulted from anhydrous ammonia theft (Fitzgerald *et al.*, 2006).

## The veterinarian and the Drug Enforcement Administration (DEA)

Title 21 of the U.S. Code of Federal Regulations establishes the DEA, which classifies drugs into five categories as listed in Table 29.1. Because some veterinary drugs, such as ketamine and phenobarbital, are coveted on the illegal drug market, failure of the veterinarian to prescribe drugs appropriately has led to loss of license and risk of imprisonment (Gloyd, 1982). A veterinarian was fined and lost his license in the year 2000 for selling steroids illegally (AVMA News, October 15, 2000) and a few years later, an Internet pharmacy was fined more than \$40,000 for contracting veterinarians to write prescriptions without examining animals and for dispensing drugs not approved by the FDA (AVMA News, April 19, 2002).

## Depressants

Substances that have a depressant effect on the central nervous system (CNS) include marijuana, barbiturates, opioids and club drugs flunitrazepam (Rohypnol®), and GHB. Ketamine, a sedative-hypnotic, will be discussed with related compound phencyclidine under the category of hallucinogens. Depressants act at a variety of receptor sites within the CNS. Some receptors are named for the drugs that bind to them, including opioid receptors and cannabinoid receptors.

## Marijuana

*Cannabis sativa* has been used for over 4000 years for its psychotropic effects (Di Marzo and De Petrocellis, 2006). Recent surveys of U.S. students found that approximately 33–37% have used marijuana by the last year of high school and 11% have used it in 8th grade (Eaton *et al.*, 2010; Latimer and Zur, 2010). Common names include marijuana, hemp or Indian hemp, pot, ganja, dagga, hashish and kief (Burrows and Tyrl, 2001). The crude product, usually called marijuana in the U.S., is produced from dried chopped leaves and the female inflorescences (Kisseberth and Trammel, 1990; Frohne and Pfänder, 2004; Volmer, 2005). Sinsemilla, Spanish for "without seeds," accounts for approximately 85% of U.S. marijuana production due to the absence of seeds and the very high content of the active ingredient. Hashish is a dried and compressed resin made from the *C. sativa* often formed into balls, sheets and cakes to be used for

TABLE 29.1 DEA drug schedules

Classification	Description	Examples
Schedule I	1. High potential for abuse. 2. No accepted medical use in U.S. 3. Lack of accepted safety for use under medical supervision.	Heroin LSD Marijuana  MDMA Mescaline Psilocybin
Schedule II	1. High potential for abuse. 2. Currently accepted medical uses, may have restrictions. 3. Severe physical or psychological dependence potential with abuse.	Amphetamine Cocaine  Methamphetamine  Morphine Opium Pentobarbital Phencyclidine
Schedule III	1. Less potential for abuse than Schedule I or II drugs. 2. Currently accepted medical uses. 3. Moderate or low potential for physical or psychological dependence with abuse.	Ketamine  LSA Thiopental
Schedule IV	1. Low potential for abuse relative to Schedule III. 2. Currently accepted medical use. 3. Limited physical or psychological dependence potential with abuse.	Butorphanol Diazepam Flunitrazepam
Schedule V	1. Low potential for abuse compared to Schedule IV. 2. Currently accepted medical use. 3. Limited potential for physical or psychological dependence with abuse compared to Schedule IV.	Low-dose codeine preparations Low-dose opium preparations

smoking. Hashish oil, even more concentrated, may be mixed with tobacco or marijuana and smoked.

Cannabinoids are a variety of related compounds found in *C. sativa*. More than 60 are known, the most important being  $\Delta^9$ -tetrahydrocannabinol (THC). THC is a lipid soluble monoterpene present in all parts of the plant, with highest concentrations in the flowers and leaves (Ashton, 2001; Burrows and Tyrl, 2001). Common cultivars grown in 1974 contained approximately 1% THC, whereas the recent average THC concentration in seized marijuana samples was 10.1% (Anonymous, 2011). Hashish contains 5% THC on average, and hashish oil averages 15% THC and may be added to a marijuana cigarette to double the potency.

There are a variety of accepted and controversial medical uses for marijuana and its active compound. Some states allow possession and use of marijuana for medical treatment under certain conditions. Though laws change frequently, states that allow restricted medical use of marijuana as of February 2011 include Alaska, Arizona, California, Colorado, Hawaii, Maine, Michigan, Montana, Nevada, New Jersey, New Mexico, Oregon, Rhode Island, Vermont, Washington and the District of

Columbia. Maryland restricts court penalties for marijuana possession if medical necessity can be proven. Prescription products include dronabinol (Marinol<sup>®</sup>) capsules, which contain THC in sesame oil and are currently classified as a Schedule I drug by the DEA, and Nabilone (Cesamet<sup>®</sup>), a synthetic form of THC classified as Schedule II. These drugs are used to treat nausea in cancer patients and may be superior in efficacy to other antiemetic drugs such as metoclopramide. They improve weight gain among patients with AIDS, cancer or Alzheimer's disease, and have been considered for use in treatment of glaucoma, multiple sclerosis, chronic pain, epilepsy and various psychiatric disorders (Di Marzo and De Petrocellis, 2006). The recently developed drug 1-pentyl-3-(1-naphthoyl)indole, or JWH-018, though not structurally related to THC, has a similar action at cannabinoid receptors. JWH-018 is mixed with leafy herbs and sold as "spice" or "K2" to be smoked as an alternative to marijuana. These products are illegal in some countries and have been temporarily banned (through the year 2011) in the United States (Anonymous, 2011).

Illegal marijuana is most commonly used in the form of marijuana cigarettes called "joints" or "reefers."

Alternately, marijuana may be rolled in a tobacco leaf and called a "blunt" or smoked using a water pipe ("bong") or other type of pipe. Marijuana may be brewed into a tea, or more commonly baked into brownies, cookies or cakes (Ashton, 2001; Volmer, 2005). Many veterinary exposures come from the ingestion of tainted baked goods.

Marijuana cigarettes are sometimes dipped in a mixture of phencyclidine, methanol and formaldehyde. Ketamine, opium, cocaine and heroin may be added to a marijuana cigarette or used with marijuana. Street names for marijuana include "grass," "hemp," "Mary Jane" or "MJ," "pot," "puff" and "weed."

Dogs less than 1 year of age are the most likely companion animal to ingest marijuana (Kisseberth and Trammel, 1990; Janczyk *et al.*, 2004). Marijuana leaves or marijuana cigarettes were recovered from 203 of 213 canine ingestions. Baked goods, as noted, are attractive to dogs (Jones, 1978; Godbold *et al.*, 1979; Janczyk *et al.*, 2004). Fresh plants and refined resins are also occasionally available to companion animals. Intentional intoxication of small animals using second-hand smoke has been reported (Schwartz and Riddle, 1985; Buchta, 1988; Frohne and Pfänder, 2004).

Large animals have been exposed through grazing marijuana or ingesting dry baled marijuana as hay (Cardassis, 1951; Driemeier, 1997). Horses have been bedded on hemp fiber, which presents an impaction risk (Green, 1996; Smith and Papworth, 1996).

### Toxicity

Few veterinary deaths have been reported from marijuana intoxications. Marijuana has a very wide safety margin in that the lethal dose is approximately 1000 times the effective dose (Volmer, 2005). No deaths were reported in dogs and monkeys ingesting 3 to 9 grams marijuana per kg of body weight (Burrows and Tyrl, 2001). Janczyk *et al.* (2004) documented the survival of a dog that ingested 26.8g marijuana/kg body weight. The LD<sub>50</sub> for oral marijuana exposure in rats is 666 to 1000mg/kg (Kisseberth and Trammel, 1990; Burrows and Tyrl, 2001). Driemeier (1997) reports that four out of five debilitated cattle died after the group ingested 35kg of dried marijuana. Horses and mules have died after ingesting large quantities of fresh marijuana of the species *Cannabis indica* (Cardassis, 1951).

### Toxicokinetics

Absorption of inhaled THC approaches 50% (Burrows and Tyrl, 2001; Janczyk *et al.*, 2004). Gastrointestinal absorption is erratic in humans and dogs. Blood concentrations obtained by ingestion are 25 to 30% of those obtained by smoking in humans (Ashton, 2001). Onset of clinical signs is delayed compared to the 6 to 12 minutes

associated with the respiratory route to 30 to 60 minutes or longer after ingestion.

Circulating THC is up to 99% protein bound in humans (Volmer, 2005). Plasma concentrations peak within 2 to 3 hours. This lipid soluble compound is rapidly distributed to the brain and other tissues. Within the brain, THC accumulates in the neocortical, limbic, sensory and motor areas. Distribution is blood flow dependent and peak accumulation in adipose tissue occurs in 4 or 5 days in humans (Ashton, 2001). The plasma half-life of THC is short due to the rapid tissue distribution.

THC is rapidly metabolized by the mixed-function oxidase system of the liver (Burrows and Tyrl, 2001). The significant first-pass effect may account for the lower blood concentrations associated with ingestion versus inhalation (Ashton, 2001; Janczyk *et al.*, 2004). 11-Hydroxy- $\Delta^9$ -THC is the physiologically active major metabolite of THC (Volmer, 2005). There are more than 20 other known metabolites (Ashton, 2001).

Between 65 and 90% of a dose of THC is excreted as the parent compound or conjugated metabolites through the feces and there may be significant enterohepatic cycling (Kisseberth and Trammel, 1990; Ashton, 2001; Volmer, 2005). Ten to 25% of THC is excreted as the parent compound, metabolites and conjugates in the urine.

### Mechanism of action

CB1 and CB2 are the cannabinoid receptors that have been identified in rats, guinea pigs, dogs, monkeys, pigs and humans (Ashton, 2005). CB1 is widely distributed in certain areas of the brain: receptors in the cerebral cortex regulate cognitive function, receptors in the hippocampus and amygdala are important in emotional status, cerebellar receptors influence dopaminergic signaling, movement and postural reflexes, and receptors in the basal ganglia, brainstem and autonomic nervous system (ANS) regulate pain perception and cardiovascular and gastrointestinal function (Ashton, 2005; Di Marzo and De Petrocellis, 2006). CB1 receptors are located within lipid membranes of presynaptic neurons and coupled to G-proteins. They inhibit cAMP and stimulate mitogen-activated protein kinases to modulate control of ion channels, particularly voltage-activated calcium ion channels and potassium channels (Ashton, 2005; Di Marzo and De Petrocellis, 2006; Janczyk *et al.*, 2004). The end result is inhibition of release of neurotransmitters, both excitatory and inhibitory. CB1 receptors also activate phospholipase C and PI-3-kinase. The endogenous ligand for cannabinoid receptors, known as endocannabinoids, are derived from arachidonic acids and closely related to prostaglandins. CB2 receptors are absent in the CNS but found in the peripheral nervous system (PNS) and immune system where they play a part in inflammation and pain regulation (Di Marzo and De Petrocellis, 2005;



Volmer, 2005). CB2 receptors regulate ceramide biosynthesis (Di Marzo and De Petrocellis, 2006).

### Clinical signs

Clinical signs of marijuana intoxication in dogs are similar to those in humans (Dumonceaux and Beasley, 1990; Dumonceaux, 1995). Signs are evident 30 to 60 minutes after ingestion. The signs attributable to the CNS include depression, ataxia, mydriasis, disorientation, behavioral disturbances, hyperesthesia, recumbence, tachycardia, hypotension or less commonly, stupor, tremors or seizures. Ingestion exposures may cause mild gastrointestinal irritation and vomiting. Other signs that have been reported include hypothermia or, less commonly, hyperthermia, as well as bradycardia, vocalization and compulsive eating. Severe clinical signs described in a ferret included ataxia with rapid onset of coma, muscle twitching, hypotension and hypothermia (Smith, 1998). A case of atopic dermatitis was reported in a dog living in a home where *C. sativa* had been cultivated (Evans, 1989).

Onset of clinical signs in cattle began 20 hours after ingesting dried plant material and included muscle tremors, hypersalivation and mydriasis. Animals were reluctant to move and lacked coordination. Four of the five exposed animals died within 3 days, one recovered with no treatment. These cattle were already debilitated at the time of exposure (Driemeier, 1997). Rapid onset of clinical signs was described in eight horses and seven mules ingesting fresh plant material, including dyspnea, tremors, hypothermia, hypersalivation, sweating, recumbence and death within 30 minutes (Cardassis, 1951).

### Treatment

The prognosis for full recovery in small animals exposed to marijuana is excellent with proper treatment. Janczyk *et al.* (2004) described 213 cases with 100% survival. Rate of recovery is dependent on dose and route of exposure. Most animals exposed to second-hand smoke recover within a few hours. Dogs who ingest a small dose of plant material usually recover within 24 hours, but those ingesting large doses can have clinical signs for several days (Kisseberth and Trammel, 1990; Dumonceaux, 1995; Burrows and Tyrl, 2001; Volmer, 2005).

Treatment for THC exposure includes decontamination, supportive and symptomatic care. Mild intoxication usually requires only observation. Gastrointestinal decontamination to decrease THC absorption can be used in animals that have ingested large quantities of marijuana or more concentrated marijuana products. Emesis may be initiated in the asymptomatic patient within an hour of ingestion, but must be avoided in animals with clinical signs such as CNS depression (Dumonceaux and Beasley, 1990; Dumonceaux, 1995). Repeated dosing with activated charcoal and cathartics helps to prevent absorption and enterohepatic cycling

and thus decreases the duration of clinical signs. THC is highly lipid soluble and, in the author's experience, intravenous lipid infusion can be used to diminish clinical signs of the THC toxicosis in dogs (unpublished information).

Observation of the patient includes monitoring cardiac function, body temperature and respiration. Stuporous or comatose dogs are at risk for respiratory suppression or hypothermia and must be treated appropriately. Marked central nervous stimulation can be treated with diazepam.

Treatment of large animals was not attempted in the few cases presented in the literature. The rapid onset of clinical signs in the horses did not allow time for veterinary intervention. Basic treatment procedures in large animals parallel those used in small animals. Gastrointestinal decontamination for large ingestions can involve gastric lavage or, in cattle, rumenotomy, and intragastric or intraruminal instillation of mineral oil or activated charcoal and cathartics. Monitoring and symptomatic and supportive care should proceed as above.

Veterinary laboratories may test blood or plasma for THC using thin-layer chromatography (TLC) or gas chromatography/mass spectrometry (GC/MS). Drug testing kits are available over the counter from pharmacies, but there is a poor correlation between blood or plasma THC levels and clinical signs (Ashton, 2001; Janczyk *et al.*, 2004).

### Pathology

There is little information on lesions associated with marijuana overdose in small animals because so few have died. Pulmonary edema was noted in a cow (Driemeier, 1997). *C. indica* was identified in the stomach of the horses with edema and petechiation of the gastric wall and myocardial hemorrhage (Cardassis, 1951).

### Barbiturates

Barbiturates are derived from the non-sedative barbituric acid. They are bitter tasting white powders in the pure form and are most frequently available as a sodium salt in weakly acidic aqueous solution. Barbiturates have been used in anesthesia and sedation and seizure control, and though still commonly used by veterinarians, are becoming more uncommon in human medicine (Kisseberth and Trammel, 1990). The four classifications of barbiturates are based on the duration of their activities. The duration of ultra-short-acting barbiturates in general is approximately 20 minutes; these drugs are given IV to effect. Examples include thiamylal sodium and thiopental sodium, both Schedule III, and methohexital sodium which is Schedule IV. The duration of short-acting barbiturates is approximately 3 hours and they are given intravenously for anesthesia. Common

examples of these are pentobarbital sodium and secobarbital sodium, both Schedule II drugs. The duration of intermediate-acting barbiturates, such as butobarbital or amobarbital, both Schedule III, is 3 to 6 hours. Long-acting barbiturates can produce clinical effects for 12 hours and these drugs have use in sedation and anticonvulsant therapy. Phenobarbital, methylphenobarbital and barbitol sodium are examples of long-acting barbiturates and are Schedule IV drugs (Kisseberth and Trammel, 1990; Branson, 2001; Volmer, 2005). Barbiturates are known as downers, reds, Christmas trees and dolls on the illegal market.

Barbiturate overdose is usually iatrogenic or due to accidental ingestion of prescription or illicit drugs. A common problem in veterinary medicine is exposure to carcasses of animals that were euthanized with barbiturates. This problem has been diagnosed by the author and reported in dogs and in wildlife in the literature (Humphreys *et al.*, 1980; Branson, 2001; Volmer, 2005). According to the AVMA News in 2003, at least 34 bald eagles have died from pentobarbital poisoning. Veterinarians and animal owners are responsible for proper carcass disposal and may be liable for wildlife poisonings (Volmer, 2005).

### Toxicity

The LD<sub>50</sub> for pentobarbital in the dog is 40 to 60mg/kg by the intravenous (IV) route or 85mg/kg per os. The oral LD<sub>50</sub> for cats is 125mg/kg. The margin of safety for barbiturates is low; the therapeutic dose may be 50 to 70% of the LD<sub>50</sub> (Kisseberth and Trammel, 1990; Branson, 2001).

### Toxicokinetics

Gastrointestinal absorption of barbiturates is variable, with rapid absorption of short-acting compounds and slower absorption of long-acting barbiturates. Barbiturates are rapidly distributed throughout the body and readily cross the blood-brain barrier (Kisseberth and Trammel, 1990; Branson, 2001; Volmer, 2005). Short-acting thiobarbiturates are highly lipid soluble and enter the brain rapidly, resulting in rapid onset of CNS depression. This is followed by rapid redistribution to tissues with less perfusion, and thus rapid clinical recovery. Longer-acting barbiturates are less lipophilic, therefore they enter and leave the brain more slowly and have a more gradual onset and longer duration of action. Barbiturates cross the placenta and fetal concentrations equilibrate with those of the dam within minutes.

There is significant variation in barbiturate metabolism and excretion based on the barbituric acid derivative, the species of animal and the individual. Barbiturates are metabolized by microsomal P450 enzymes in the liver. Barbiturates interfere with metabolism of other compounds either by binding to the P450 enzymes to block

metabolism or, in chronic exposures, by inducing P450 enzymes and increasing the rate of metabolism of xenobiotics and endogenous substances, including steroids (Volmer, 2005). Some barbiturates, in particular the short-acting thiobarbiturates, undergo significant oxidation in the tissue. Phenobarbital is metabolized very rapidly in ruminants and horses, relatively rapidly in dogs, more slowly in humans, and slower still in cats (Branson, 2001).

Barbiturates are excreted as both parent compound and metabolites in the urine. Excretion of some barbiturates is dependent on urine pH and is increased five to ten times in alkaline urine through ion trapping. Ion trapping is less effective with short-acting barbiturates, which are highly metabolized, highly protein bound drugs, and have high pK<sub>a</sub>s (Kisseberth and Trammel, 1990; Volmer, 2005). Barbitol is excreted very slowly in birds (Branson, 2001).

### Mechanism of action

Barbiturates bind to the  $\gamma$ -aminobutyric acid (GABA) receptor complex and decrease the rate of GABA dissociation (Branson, 2001). These actions increase permeability of the postsynaptic membrane to chloride, causing membrane hyperpolarization and reduced excitability. GABA receptors are found in motor and sensory areas of the cerebral cortex and possibly in the thalamus, where barbiturates act to control seizures and induce anesthesia. Barbiturates also act to inhibit glutamate receptors and decrease norepinephrine (NE) release (Volmer, 2005). Actions on the peripheral nervous system (PNS) include inhibition of acetylcholine sensitive nerve depolarization at postsynaptic junctions and motor end plates. Respiratory depression is caused by chemoreceptor suppression by barbiturates. This effect is more marked in cats, where the reticular formation governs medullary control of respiration. Control of respiratory activity is believed to be more complex in other species.

Barbiturates cause hypotension and secondary anuria (Branson, 2001; Volmer, 2005). Increased heart rate is believed to be governed by arterial pressoreceptors. The effects of barbiturates on levels of sodium, potassium and calcium in cardiac myocytes produce a change in cardiac contractility. Hypothermic animals are predisposed to ventricular fibrillation with barbiturates. Fibrillation is reported in 100% of hypothermic animals given pentobarbital and 50% of those given thiopental.

### Clinical signs

Onset of clinical signs depends on the route of exposure, the barbiturate involved and the presence or absence of food in the stomach. Animals that have ingested short-acting barbiturates usually have clinical signs within 30 minutes. If long-acting barbiturates were ingested, the first effects might not be observed for an hour

(Kisseberth and Trammel, 1990). The time to onset of clinical anesthesia is doubled if phenobarbital is given to an animal with food in the stomach versus an empty stomach, or if contaminated carrion is ingested. Duration of effects is also variable, as alluded to previously, and dependent on the agent involved, species of animal, nutritional status, age, sex and weight. The duration of a given barbiturate is expected to be prolonged in greyhounds, which have less adipose tissue for the drug deposition.

The predominant signs of barbiturate intoxication are profound CNS depression and anesthesia (Humphreys *et al.*, 1980; Kisseberth and Trammel, 1990; Volmer, 2005). Animals usually present with severe ataxia, weakness, disorientation and loss of deep tendon reflexes. Hypothermia is common and is associated with cardiac dysrhythmia in humans and dogs. The pulse is rapid and weak and severe respiratory depression may lead to cyanosis and death.

Certain preexisting conditions enhance the effects of barbiturates such as renal failure, uremia and decreased rate of excretion. Barbiturates can accelerate liver damage in an individual with preexisting liver disease. Allergic reactions to barbiturates have been reported.

### Treatment

Decontamination for recent exposures, monitoring and symptomatic and supportive therapy are the basis of treatment for barbiturate overdose. Respiratory function, cardiac function and body temperature are closely monitored (Kisseberth and Trammel, 1990; Branson, 2001; Volmer 2005). Emetics are given only to the *asymptomatic* animal if it is soon after ingestion. Gastric lavage after intubation is more appropriate in the animal presenting with CNS depression. Repeated doses of activated charcoal function to decrease the biological half-life of barbiturates. A cathartic such as sorbitol can be added, but magnesium-containing cathartics such as magnesium sulfate (Epsom salt) should be avoided because they can enhance CNS depression. Use of intravenous lipid emulsion would be likely to decrease the bioavailability of highly lipid soluble barbiturates and minimize clinical signs.

Monitoring and control of body temperature is essential to prevent ventricular fibrillation and decrease the duration of the clinical signs. Intubation and assisted ventilation are required when marked respiratory suppression is noted. Fluid therapy is often needed to maintain cardiac and renal function. Alkaline diuresis has been used to increase the rate of excretion of some barbiturates, but is ineffective with short-acting barbiturates. Because tissue partitioning attenuates the effects of barbiturates, it is possible for the patient to relapse as the drug repartitions from the tissues back into plasma. The use of some drugs is contraindicated in the barbiturate

overdose patient. Dextrose, fructose, lactate, pyruvate and glutamate can increase partitioning of barbiturates into the central nervous system. Epinephrine and isoproterenol cause re-anesthetization after thiopental exposure. Sulfonamides, salicylates and doxycycline increase bioavailability of barbiturates by displacing them from plasma proteins.

Many laboratories can analyze samples such as urine, blood products or tissues for barbiturates. Common techniques include TLC and GC/MS. Over-the-counter drug testing kits for urine have been successful in the detection of barbiturates in canine urine in one study, though these kits are not always reliable in one author's experience (Teitler, 2009).

### Opioids

Opium is produced from *Papaver somniferum*, a poppy, and its use was recorded in the Ebers Papyrus, one of the oldest known medical texts dated around 1500 BC. The unripe seed capsule is incised after the petals have fallen. Material that exudes from the capsule is dried, collected and dried further to produce opium. Powdered opium is 75% inert ingredients, about 10% morphine, the major active alkaloid, 0.5% codeine, and the third major alkaloid is dimethyl-morphine or thebaine, which acts as a convulsant (Branson and Gross, 2001). Laudanum is deodorized tincture of opium and paregoric is camphorated tincture of opium.

Morphine sulfate is a Schedule II drug commonly used medicinally for pain control and sedation. Oxymorphone is a Schedule II morphine derivative approved for use in dogs and cats in the U.S. Oxymorphone is approximately ten times more potent than morphine (Branson and Gross, 2001). Other morphine derivatives include codeine and the more potent hydromorphone, both Schedule II drugs. Heroin is a Schedule I morphine derivative.

Synthetic opioids include methadone, propoxyphene, meperidine and pentazocine, all Schedule III drugs. Butorphanol is Schedule IV. Oxycodone and hydrocodone are common prescription synthetic opioids classified as Schedule II. Fentanyl is formulated into injectable solutions, slow-dissolving sticks for oral transmucosal absorption and patches that release doses of 25, 50, 75 or 100 µg per hour for transdermal absorption. Fentanyl is about 80 times more potent than morphine (Branson and Gross, 2001). Other potent synthetic opioids are alfentanil, sufentanil citrate and etorphine HCl, which is 10,000 times as potent as morphine and used in wildlife capture. These potent drugs are classified in Schedule II.

The morphine derivative heroin is one of the most abused opioids, and use is increasing, due to increased availability and relatively low cost, compared to

prescription opioids (Dumonceaux, 1995; Anonymous, 2011). A recent survey of U.S. high-school students found that 2.5% have used heroin, though another survey stated that only about 1% had used heroin in the past year and up to 25% said it was accessible to them (Latimer and Zur, 2010). A white to dark brown powder, heroin often contains additives such as sugar, starch, powdered milk, quinine or strychnine. Heroin was traditionally injected but insufflation (snorting) and smoking are becoming more popular. Heroin is also available as a black sticky substance termed "black tar heroin," which is dissolved, diluted and injected.

Oxycodone, sold as OxyContin®, Percocet® and in a generic form, is commonly used recreationally. A recent survey found that 9% of U.S. high-school seniors had used prescription opioids recreationally (Latimer and Zur, 2010). Pills are time-released but may be crushed and insufflated or injected in solution. Hydrocodone, sold under various trade names and in formulations with acetaminophen (Vicodin®) is also commonly sold on the illegal drug market. Fentanyl can be produced in clandestine laboratories, and is used intravenously.

Small animal exposure to opioids may be due to accidental ingestion of pharmaceuticals, illegal drugs or occasionally by oral or parenteral dosing with malicious intent (Kisseberth and Trammel, 1990; Volmer, 2005).

### Toxicity

With such a wide variety of opioids, there is much variation in toxicity. The lethal parenteral dose of morphine in dogs is between 110 and 210 mg/kg (Kisseberth and Trammel, 1990; Branson, 2001; Volmer, 2005). The minimal lethal dose for subcutaneous injection of morphine in the cat is 40 mg/kg. The minimum lethal dose of heroin for the dog is 25 mg/kg given subcutaneously. Approximately 0.20 mg/kg heroin causes clinical signs in dogs, including sedation and respiratory depression, whereas a dose of 0.58 mg/kg causes increased duration of effects, respiratory difficulty and aggressive behavior with clinical signs lasting up to 8 hours (Garret and Gürkan, 1980). The minimum oral lethal dose of heroin for the cat is 20 mg/kg. Meperidine causes clonic convulsions in cats at a dose of 30 mg/kg (Branson and Gross, 2001).

### Toxicokinetics

Opioids are weakly acidic and absorption after oral dosing is predominantly in the small intestine. There is rapid absorption from the subcutis after subcutaneous injection (Branson and Gross, 2001). Distribution is variable. Heroin is more lipophilic than morphine and more readily crosses the blood-brain barrier (Garrett and Gürkan, 1980). Opioids are also distributed to skeletal muscle, kidney, liver, intestine, lungs, spleen, brain and placenta

(Kisseberth and Trammel, 1990). Opioids are readily metabolized in the liver with a significant first pass effect after ingestion. Phase I metabolism includes hydrolysis, oxidation and N-dealkylation. Approximately 50% of a dose of morphine is conjugated to glucuronide in most species. A notable exception is the cat, accounting in part for the increased sensitivity of this species. The primary glucuronide conjugate is morphine-3 glucuronide, though the metabolically active morphine-6-glucuronide is also produced. Heroin is metabolized in the liver and other tissues and is cleared more rapidly than morphine. Deacetylation produces 6-*o*-acetylmorphine and morphine, which appear in the plasma of dogs within minutes (Garrett and Gürkan, 1980).

Opioids are excreted predominantly as metabolites in the urine. Metabolites may be detected in horse urine for up to 6 days (Branson and Gross, 2001). There is some biliary excretion and enterohepatic cycling. The biological half-life of morphine in cats is about 3 hours. The initial plasma half-life of heroin in dogs is 8 minutes, but the terminal half-life is 80 minutes due to repartitioning from the tissues (Garrett and Gürkan, 1980).

### Mechanism of action

Major opioid receptors are designated  $\mu$ ,  $\delta$  and  $\kappa$  and are believed to be stimulated by endogenous endorphins (Branson and Gross, 2001; Volmer, 2005). The  $\mu$  receptors are activated by endogenous enkephalins. These receptors may be further differentiated into  $\mu_1$  and  $\mu_2$ . Activation of the  $\mu_1$  receptor induces supraspinal analgesia and  $\mu_2$  receptor activation causes spinal analgesia, suppression of respiration and inhibition of gastrointestinal motility. The  $\delta$  receptors are more selective for enkephalins than the  $\mu$  opioid receptors and are involved in spinal analgesia. Activation of  $\kappa$  receptors produces spinal and supraspinal analgesia, sedation and dysphoria. The  $\sigma$  receptor, previously classified as an opioid receptor, does not appear to mediate analgesia.

Opioid receptors are found in the CNS, autonomic nervous system (ANS), gastrointestinal tract, heart, kidney, pancreas, adrenal glands, vas deferens and on the surface of lymphocytes and adipocyte (Volmer, 2005). Opioid receptors in the CNS are concentrated in the amygdala and frontal cortex in the dog, monkey and human where stimulation is associated with CNS depression (Branson and Gross, 2001; Kisseberth and Trammel, 1990). Activation in the cat, swine, goat, sheep, horse or ox may alter dopaminergic or noradrenergic function, leading to excitation. Activation of opioid centers in the chemoreceptor trigger zone in the fourth ventricle of the brain produces emesis. Opioid receptors are present in the brain stem, including the cough centers where they suppress the cough reflex, and the respiratory centers, though initial increases in respiration are



followed by respiratory suppression due to generalized CNS depression. Stimulation of gastrointestinal opioid receptors decreased motility, leading eventually to delayed passage of ingesta, increased water absorption and constipation.

Opioid drugs act as agonists, partial agonists or antagonists. Morphine and related drugs are agonists at  $\mu$  receptors. Naloxone is an antagonist with a high affinity for the  $\mu$  receptors and a low affinity for  $\delta$  receptors (Volmer, 2005). An opioid is classified as a partial agonist if it acts as an agonist at one receptor but an antagonist at another.

### Clinical signs

The clinical effects of opioid drugs are dependent on age (as neonates have an incomplete blood-brain barrier), species and the drug involved. Clinical signs in dogs are similar to those described in humans. Early clinical signs can include transient excitation and rapid respiration, drowsiness and ataxia, decreased pain perception, hypersalivation, vomiting, defecation and urination. Dogs progress to stupor or coma with hypothermia, hypotension, respiratory depression and death (Kisseberth and Trammel, 1990; Dumonceaux, 1995; Branson and Gross, 2001; Volmer, 2005). Other signs include decreased urination and constipation. Signs in dogs given low doses of heroin included early aggressive behavior, brief unconsciousness, weakness, hypersalivation and respiratory difficulty that lasted up to 8 hours (Garret and Gürkan, 1980). Propoxyphene can induce tremors and convulsions in dogs. Cats may present with excitation, aggression, insomnia and hyperthermia as well as increased pain threshold. Cats are highly resistant to the emetic effects of opioids. Horses, ruminants and swine also exhibit CNS stimulation. Clinical signs in rabbits depend on the drug used: morphine induces hypothermia whereas apomorphine induces hyperthermia.

### Treatment

The basis of treatment for opioid exposure is early decontamination, symptomatic and supportive care, and the judicious use of opioid antagonists. Induce vomiting in animals presenting immediately after ingestion of narcotics if they have not already vomited and no contraindications exist. If large doses are ingested and contraindications against emetic use are present, enterogastric lavage on the anesthetized and intubated animal can be performed to evacuate the stomach and instill activated charcoal and cathartics. Decontamination is likely to be effective for several hours after ingestion due to decreased gastrointestinal motility. Animals must be monitored closely for respiratory dysfunction, the most common cause of death in opioid overdoses. Assisted

ventilation is often necessary. Maintain body temperature and treat seizures with diazepam.

The opioid antagonist Naloxone<sup>®</sup> is used to treat severe CNS or respiratory depression. Naloxone can be given parenterally at a dose of 0.01 to 0.02 mg/kg and repeated as necessary, since it has a very short half-life. Patients that fail to respond to a 10 mg dose of Naloxone<sup>®</sup> are unlikely to respond to a higher dose.

Testing of urine or plasma for opioids is available at some diagnostic laboratories. Over-the-counter drug tests kits have been used with variable success to detect opioids in canine urine (Teitler, 2009).

### Other CNS depressants

Other drugs may have depressant activities on the central nervous system. Two commonly used "club drugs" are discussed below: flunitrazepam and GHB. Both are often used by assailants to sedate potential victims, thus there is potential for their use on guard dogs.

#### Flunitrazepam

Flunitrazepam is not sold in the United States but is available in more than 60 countries, including many in Europe and Latin America, as Rohypnol<sup>®</sup>, Narcozep<sup>®</sup>, and under other trade names, as a sedative. Rohypnol<sup>®</sup> has gained notoriety as the "date rape drug" and is sold as tablets imprinted "Roche 1" and "Roche 2" designating their manufacturer and milligram dosage. Tablets may be crushed and insufflated or dissolved in a drink intended for an unsuspecting victim (Smith *et al.*, 2002; Anonymous, 2011). Blue coloring has been added to these pills to reduce the potential for this use (Smith *et al.*, 2002; Rimsza and Moses, 2005). Street names include "roofies" or "rophies," "Roche" or "la rocha," "Mexican valium," "forget me pills," "Rope" and "R2." Injectable forms of flunitrazepam are also available.

Animals that have ingested flunitrazepam should be monitored closely for respiratory and CNS depression, changes in heart rate and body temperature fluctuations. If ingestion was recent and there is not yet evidence of CNS depression, emetics can be given followed by activated charcoal and cathartics to help minimize absorption. Gastric lavage can be performed on the intubated patient who has ingested a large dose and presents with severe CNS depression. Analysis is available at some laboratories for flunitrazepam, but doses are often quite low and urine must be collected soon after ingestion.

#### GHB

Another popular "club drug" that has been associated with date rape is GHB. This compound is a derivative of GABA and is present in the body under normal

conditions. Synthetic GHB can be produced in clandestine laboratories. It was sold as a nutritional supplement for body builders at one time, and is currently used to treat narcolepsy. Industrial solvents  $\gamma$ -butyrolactone (GBL) and 1,4-butanediol (BD) are metabolized to GHB. BD is available in printer ink cartridges and has been sold as a supplement at health food stores (Smith *et al.*, 2002; McDonough *et al.*, 2004; Anonymous, 2011). BD was also used as a substitute for 1,5-pentanediol in the production of a children's craft product called "Aqua Dots" in the U.S. and "Bindeez" in Australia (Suchard *et al.*, 2009). The substitution was made in 2007 and resulted in clinically affected children. GHB is most commonly sold as a clear liquid in small vials and added to bottled water (Smith *et al.*, 2002; Rimsza and Moses, 2005). Street names include "Liquid X" or "liquid ecstasy," "soap" or "salty water" due to the flavor of the product, "easy lay," "Georgia homeboy," "grievous bodily harm," "G," "goop," "gib" or "scoop."

GHB receptors are present in the hippocampus, cortex and other areas of the brain (McDonough *et al.*, 2004). GHB is involved in regulation of sleep cycles, body temperature, memory, glucose metabolism and dopamine levels (Smith *et al.*, 2002). GHB can also be converted back to GABA. Human death has been reported at an oral GHB dose of 5.4 g, but some patients have survived doses of 29 g. The LD<sub>50</sub> for rodents and rabbits is approximately 2 g/kg. GHB is rapidly absorbed from the gastrointestinal tract and, because it is highly lipophilic, it readily crosses the blood-brain barrier. GHB is rapidly metabolized to carbon dioxide though 2 to 4% of a given dose can be excreted in the urine (Smith *et al.*, 2002; Gable, 2004). Half-life is dose dependent.

Clinical signs attributed to GHB usually occur within half an hour of ingestion and include euphoria, reduced anxiety and drowsiness. CNS depression sometimes progresses to loss of motor control, unconsciousness and respiratory depression (Smith *et al.*, 2002; McDonough *et al.*, 2004; Rimsza and Moses, 2005). Clinical signs reported in children ingesting "Aqua Dots" were vomiting, ataxia and coma (Suchard *et al.*, 2009). Seizures have been reported, and bradycardia and hypothermia are reported in nearly a third of overdose patients.

Treatment consists of decontamination of the intubated patient with either gastric lavage, after large ingestion, or activated charcoal – *emetics are contraindicated* due to the rapid onset of CNS depression – and close monitoring of respiratory, cardiovascular and CNS function, and body temperature. Supportive care is instituted based on clinical signs. Seizures have been treated with benzodiazepines. Patients who appear stable should be monitored for at least 8 hours (Smith *et al.*, 2002). GHB is difficult to analyze for and results are difficult to interpret because this compound is rapidly metabolized and normally present in the body.

## STIMULANTS

The major illicit drugs classified as stimulants are cocaine and the amphetamines. Cocaine is a plant alkaloid and amphetamines are a large group of compounds, including prescription drugs such as methylphenidate, sold as Ritalin®, and methamphetamine, which is produced in clandestine laboratories. Stimulants in general act on the ANS, usually on adrenergic receptors. Popular "club drug" 3,4-methylenedioxymethamphetamine (MDMA), or "ecstasy," also has significant action on serotonin receptors. Various legally available compounds act as stimulants through various mechanisms. These include nicotine, caffeine and related compounds, and are discussed elsewhere in the text. Areca alkaloids, described below, are commonly used as stimulants across much of Asia and are available in some parts of the U.S. and Europe.

### Cocaine

Cocaine is the natural alkaloid of the shrubs *Erythroxylon coca* and *E. monogynum*, originally from the Andes Mountains in South America, most commonly grown in Bolivia, Peru and Columbia (Queiro-Neto *et al.*, 2002; Volmer, 2005; Anonymous, 2011). Traditionally, *E. coca* leaves are brewed into tea by those native to the Andes Mountains, who share this delightful beverage with unsuspecting tourists in order to counter the effects of the high altitude. Cocaine is a Schedule II drug used for topical anesthesia and vasoconstriction of mucous membranes (Kisseberth and Trammel, 1990). It may be second only to marijuana in illegal consumption in the U.S. (Dumoncaux, 1995; Queiro-Neto *et al.*, 2002; Vitale and van de Mheen, 2005). According to surveys in the 1980s, approximately 15% of the U.S. population had tried cocaine (Kabas *et al.*, 1990). Recent surveys of high-school students in the U.S. found that 5 to 6.4% had used cocaine (Eaton *et al.*, 2010; Latimer and Zur, 2010).

Cocaine is sold as a powdered white salt, cocaine HCl, ranging in purity from 12 to more than 60% (Kisseberth and Trammel, 1990). It is diluted with inert ingredients such as lactose, inositol, mannitol, corn starch or sucrose, or with active compounds including procaine, lidocaine, tetracaine, caffeine, amphetamine or quinine. Recently, contamination of cocaine with levamisole has been associated with severe adverse effects in users, including agranulocytosis and vasculitis (Buchanan *et al.*, 2010; Wiegand, 2010). The water soluble salt can be injected, ingested or insufflated (Rimsza and Moses, 2005; Anonymous, 2011). Common street names for cocaine HCl include "bernies," "blow," "C" or "big c," "coke," "girl" or "white girl," "gold dust" or "star dust,"

"her," "lady" or "white lady," "nose candy," "snow" or "toot."

Cocaine HCl is converted to the free alkaloid by dissolving it in a basic solution which is boiled to precipitate the alkaloid. The dry precipitate is broken into "rocks" which are 75 to 90% pure, but are often diluted with inert ingredients or active compounds such as procaine, lidocaine, amphetamines, heroin, caffeine, phencyclidine, ergot alkaloids and strychnine. The free base readily vaporizes with heat and the smoke is inhaled, though it may be taken orally. This form of cocaine is termed "crack" because of the sound produced when it is heated, but it is sometimes called "bedrock," "beamers," "BJ's," "bolo," "crank," "crystal," "flake," "ice," "jelly beans," "rock," "rooster," "space," "tornado" or "24/7."

Exposure to illicit cocaine is most likely to affect dogs, particularly police dogs, but athletic horses are sometimes dosed with cocaine to improve performance (Dumoncaux and Beasley, 1990; Kisseberth and Trammel, 1990; Frazier *et al.*, 1998; Queiroz-Neto *et al.*, 2002; Kollias-Baker *et al.*, 2003; Volmer, 2005).

### Toxicity

The LD<sub>50</sub> for cocaine in dogs is 3 mg/kg IV, and the LD<sub>99</sub> is 20 mg/kg IV. Dogs can tolerate two to four times the above doses if given PO. The minimum lethal dose in cats is approximately 7.5 mg/kg IV or 16 mg/kg subcutaneous (SC). Horses given 50 mg of cocaine IV showed no clinical signs, but performance was enhanced at 200 mg (Kollias-Baker *et al.*, 2003).

### Toxicokinetics

Cocaine is highly lipophilic and readily absorbed from all mucosal surfaces, including those of the nose, oral cavity, gastrointestinal tract, and alveoli. Approximately 20% of an ingested dose is absorbed. Peak plasma concentrations appear between 15 minutes and 2 hours after ingestion and cocaine readily crosses the blood-brain barrier. The neurological effects of cocaine and crack last for 15 to 20 minutes after insufflating or 5 to 10 minutes after smoking recreational doses.

Cocaine undergoes hydrolysis by plasma esterases to water soluble metabolites benzoylecgonine, ecgonine methyl ester and others (Queiroz-Neto *et al.*, 2002). Hepatic esterases and demethylating enzymes play a role in cocaine metabolism (Kisseberth and Trammel, 1990; Volmer, 2005). Up to 20% of a dose of cocaine is excreted unchanged in the urine. Benzoylecgonine and ecgonine methyl ester are the primary metabolites excreted in the urine of most mammals in both conjugated and unconjugated forms (Kollias-Baker *et al.*, 2003). Other metabolites include norcocaine, benzoylecgonine, norecgonine and ecgonine. Kollias-Baker *et al.* (2003) found cocaine in the urine for up to 24 hours

when horses were dosed sublingually with 2.5 mg. Larger doses are detectable for a few days.

### Mechanism of action

Cocaine increases release of catecholamines and blocks reuptake of NE, serotonin and dopamine, leading to increased neurotransmitter concentrations at synaptic junctions (Kisseberth and Trammel 1990, Volmer, 1995; Queiroz-Neto *et al.*, 2002; Vroegop *et al.*, 2009). NE regulates thalamic effects on appetite, body temperature and sleep. Cocaine also influences the endogenous opiate system. Local anesthetic actions occur through inhibition of membrane sodium ion channels.

Cardiac effects are often associated with IV dosing of cocaine (Kabas *et al.*, 1990; Kisseberth and Trammel, 1990). Cocaine acts directly on myocardium by blocking sodium ion channels, thus causing conduction disturbances and prolonged R waves. Cocaine increases calcium concentrations within cardiac myocytes and can promote depolarization during the diastolic interval leading to ventricular fibrillation. Cocaine slows conductance at the bundle of His. Oxygen demand is increased within the myocardium but constriction of the coronary vasculature leads to hypoxia and infarction (Kabas *et al.*, 1990; Volmer, 2005; Vroegop *et al.*, 2009).

### Clinical signs

Clinical signs of cocaine toxicosis are associated with CNS stimulation, sometimes followed by depression (Kisseberth and Trammel, 1990). Signs in dogs include hyperactivity, hyperesthesia, tremors, seizures and death. Other reported signs are mydriasis, hypersalivation and vomiting. Cardiac changes are consistently reported. Dogs dosed IV with cocaine had increased heart rate, cardiac output and mean arterial pressure (Catravas and Waters, 1981). Electrocardiographic changes include increased duration of QRS complexes and P-wave abnormalities (Llera and Volmer, 2006). Death was attributed to hyperthermia, secondary to increased muscular activity and peripheral vasoconstriction (Catravas and Waters, 1981; Dumoncaux and Beasley, 1990; Dumoncaux, 1995; Frazier *et al.*, 1998; Vroegop *et al.*, 2009). Frazier *et al.* (1998) reported a body temperature of 105°F (40.56°C) in one dog. Respiratory and cardiac arrest, the latter attributed to coronary vasospasm, have also been implicated as cause of death. Lactic acidosis, hypoglycemia or hyperglycemia, and elevated creatinine phosphokinase have been reported.

Queiroz-Neto *et al.* (2002) reported mild clinical signs in horses given low IV doses of cocaine: increased alertness, irritability, muscle tremors, vocalization and stereotypical behaviors like head-bobbing and pawing at the ground. Cribbing was reported at higher doses. Most horses defecated within 10 minutes of dosing and recovered within

20 minutes. Lesions reported in dogs include subendocardial and epicardial hemorrhage, degeneration of cardiac myofibers, coronary vasoconstriction, pericardial effusion and pulmonary hemorrhage.

### Treatment

Early decontamination of dogs suspected of recently ingesting cocaine has been recommended, but is likely to have limited effects because the drug is absorbed extremely rapidly (Dumonceaux and Beasley, 1990; Dumonceaux, 1995). Furthermore, emesis is likely to induce seizures (Volmer, 2005; Llera and Volmer, 2006). Sedation and gastric lavage in the patient who has ingested a large quantity of cocaine is a safer method of decontamination. Activated charcoal and a cathartic can be given orally. Police dogs that have ingested bags of cocaine require cautious endoscopic or surgical retrieval to prevent rupture or obstruction. Surgically implanted bags must be removed with equal caution and secondary infection treated as necessary. Because cocaine is highly lipophilic, intravenous infusion of lipid emulsion is likely to decrease the bioavailability and clinical effects of cocaine.

Symptomatic and supportive care includes maintaining body temperature, acid-base and electrolyte status, and monitoring cardiac and respiratory function. Body temperature may be maintained by use of a cool environment, cool fluids, cool bath, wet towels, fans or cool water enemas, but avoid inducing shivering, which will increase body temperature. Decreased stress also prevents hyperthermia.

Seizure control also prevents hyperthermia. Diazepam has been used to decrease seizure activity; however, two out of six animals in one study that had an apparent favorable response to diazepam died within 72 hours (Catravas *et al.*, 1977). Barbiturates are recommended to treat refractory seizures. Chlorpromazine given before cocaine dosing was effective experimentally to reduce severity of seizures, maintain blood pH, maintain body temperature, decrease heart rate and blood pressure, and prevent arrhythmias (Catravas and Waters, 1981). Affected animals require respiratory support, including intubation and mechanical ventilation, for severe or prolonged respiratory depression. Cardiac signs such as tachycardia are usually short-lived. Intravenous fluids with sodium bicarbonate are likely to be adequate to decrease electrocardiogram changes and risk of ventricular arrhythmia development. Treatment of *life-threatening* cardiac arrhythmia with beta-blockers such as propranolol has been recommended, but pretreatment of dogs with propranolol before cocaine injection did not increase survival. Beta-blockers are known to produce systemic hypertension (Vroegop *et al.*, 2009).

Urine and plasma are routinely tested for cocaine at many laboratories. Thin-layer chromatography and

immunoassays may be used with confirmation by gas chromatography/mass spectroscopy. Over-the-counter test kits are available to test for cocaine in urine, but their usefulness to the veterinarian has yet to be determined.

### Amphetamines

The term "amphetamine" refers specifically to  $\alpha$ -methylphenylethylamine, a Schedule II drug, but the term is often used to describe various derivatives. The term "amphetamines" (plural) is used here to describe a group of related compounds unless specified otherwise. Common amphetamines include the Schedule II drug methamphetamine and Schedule I drugs 2,5-dimethoxy-4-methylamphetamine ("DOM," "STP"), 2,5-dimethoxy-4-bromoamphetamine ("DOB"), methylphenidate, 4-methylaminorex (4MA) and 3,4-methylenedioxy-N-ethylamphetamine (MDEA). The "designer drug" 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") has some unique characteristics and is discussed separately below.

Historically, amphetamines were used by veterinarians for the stimulation of medullary respiratory centers in order to increase respiratory rate and depth in animals (Adams, 2001). These drugs are not currently used in veterinary medicine. Physicians have used amphetamines to control appetite in obese patients, to treat narcolepsy, depression, alcoholism and, counter-intuitively, to control hyperkinetic behavior in children. Most are sold as tablets or capsules, which may be sustained release (Kesseberth and Tremmel, 1990). A recent study found that 5% of 8th grade students in the U.S. and 7% of high-school students surveyed had used amphetamines in the past year (Latimer and Zur, 2010).

Amphetamines on the illegal market are sold as "bennies," "dex" or "dexies," "speed" or "uppers." Prescription products may find their way to the illegal market, but many "designer" amphetamines such as methamphetamine are created in clandestine laboratories. Crystal methamphetamine, called "ice" or "glass," may be smoked, powdered methamphetamine, termed "crank" or "meth," may be dosed intravenously, orally or insufflated (Rimsza and Moses, 2005; Anonymous, 2011). "Yaba" contains methamphetamine and caffeine. One survey found that about 4% of U.S. high-school students have used methamphetamine (Eaton *et al.*, 2010). Other "designer" amphetamines include 4-methylaminorex, sold as "euphoria," "U4EUH," or "ice," MDEA, sold as "Eve," methylcathinone (see Khat) and MDMA. Drug dealers may combine amphetamine with inert or other active ingredients, or may substitute other drugs such as heroin, cocaine or phenylethylamine.

Accidental ingestion of prescription amphetamines is the most likely exposure risk for companion animals.



Potential exists for exposure of companion animals to illegal drugs. Illegal doping may occur in horses.

### Toxicity

Catravas *et al.* (1977) found that 10 mg/kg IV amphetamine killed dogs within 3 hours. LD<sub>50</sub>s for orally administered amphetamine sulfate and methamphetamine in the dog are 20 to 27 mg/kg and 9 to 100 mg/kg, respectively (Diniz *et al.*, 2003; Volmer, 2005).

### Toxicokinetics

Absorption of amphetamines through the gastrointestinal system is usually rapid, though it is slower with sustained release products (Dumonceaux and Beasley, 1990; Kesseberth and Trammel, 1990; Dumonceaux, 1995; Volmer, 2005). Methamphetamine absorption is more rapid by insufflation with onset of clinical signs in 2 to 5 minutes, whereas the onset after PO dosing is 15 to 20 minutes (Anonymous, 2011).

Peak plasma concentrations of amphetamine occur 1 to 3 h after ingestion, unless a sustained release product was ingested. Amphetamines are highly lipid soluble and readily cross the blood-brain barrier (Volmer, 2005). Concentrations in the cerebral spinal fluid may be 80% of those found in the plasma (Kesseberth and Trammel, 1990). Methamphetamine has increased partitioning to the CNS compared to other amphetamines (Rimsza and Moses, 2005). Amphetamine is also distributed to the kidneys, liver and lungs, with negligible storage in the adipose tissue (Baggot and Davis, 1972).

There is significant hepatic metabolism of amphetamines. The two major pathways are hydroxylation and deamination. Deaminated products may be oxidized and conjugated to glycine (Baggot and Davis, 1972). Active metabolites may be produced. Amphetamine and its metabolites are excreted primarily in the urine and minimally in the bile. About 8% of an amphetamine sulfate dose is excreted unchanged in the urine in swine and 30% in dogs. Rate of excretion is significantly increased as urine pH declines (Baggot and Davis, 1972; Kesseberth and Trammel, 1990; Volmer, 2005). Amphetamine is almost completely eliminated within about 6 hours in dogs with an average urinary pH of 7.5, and in 3.3 h if the urinary pH averages around 6.0.

### Mechanism of action

Questions remain concerning the mechanism of action of amphetamines. Sympathetic central and peripheral effects are due to direct actions on  $\alpha$  and  $\beta$  adrenergic receptors, increased release of catecholamines, particularly norepinephrine, inhibition of monoamine oxidase (MAO) and inhibition of catecholamine reuptake (Kesseberth and Trammel, 1990; Adams, 2001;

Diniz *et al.*, 2003; Volmer, 2005; Llera and Volmer, 2006). Amphetamines may promote serotonin and dopamine release and act directly on dopamine receptors.

### Clinical signs

Common clinical signs of amphetamine toxicosis in animals include mydriasis, hyperactivity, restlessness, tremors and seizures, and occasionally ataxia and depression. Hyperthermia may be secondary to seizures and peripheral vasoconstriction. Tachycardia and ventricular premature contractions, hypertension, or occasionally hypotension, have also been observed. Other signs are mydriasis, hypersalivation and vocalization.

Reported causes of death in amphetamine overdosed dogs include disseminated intravascular coagulation secondary to hyperthermia and respiratory failure (Davis *et al.*, 1978; Diniz *et al.*, 2003). Cerebrovascular hemorrhages due to hypertension, hypoglycemia, lactic acidosis and cardiac failure have all been implicated as the cause of death after amphetamine overdose (Catravas *et al.*, 1977).

Serum chemistry abnormalities in addition to lactic acidosis and hypoglycemia that have been reported with amphetamine overdose include hyperkalemia, hyperphosphatemia and elevated liver enzymes such as alanine transaminase, alkaline phosphatase and aspartate transaminase (Catravas *et al.*, 1977; Diniz *et al.*, 2003; Llera and Volmer, 2006). Rhabdomyolysis is evident in the presence of increased creatine kinase, myoglobinuria, and there is evidence of renal failure (Kesseberth and Trammel, 1990; Diniz *et al.*, 2003). Lesions in experimental dogs dosed with amphetamines include subendocardial and epicardial hemorrhage and myocardial necrosis.

Low doses of amphetamine given to horses caused increases in heart rate during rest and exercise, increased blood pressure, second degree AV block and premature ventricular contractions (Smetzer *et al.*, 1972).

### Treatment

The prognosis for animals that have ingested amphetamines depends on the dose, time between exposure and presentation, and severity of clinical signs. Gastrointestinal decontamination should be initiated in animals that present within 2 h of ingestion (Kesseberth and Trammel, 1990; Dumonceaux, 1995). Rapid onset of clinical signs may preclude the use of emetics. Gastric lavage of the sedated animal may be warranted if large doses were ingested. Activated charcoal and a cathartic help prevent absorption, but repeat dosing is necessary for sustained release products.

Animals should be monitored closely for neurologic signs, hyperthermia, cardiac arrhythmias or respiratory insufficiency. Baseline serum chemistries should be taken to monitor for hypoglycemia, electrolyte

abnormalities, lactic acidosis, myoglobinuria, liver and kidney damage, etc.

Minimal external stimulation helps to prevent seizure activity. Diazepam may paradoxically exacerbate the clinical signs of many amphetamines (a possible exception is MDMA, see below) and is thus contraindicated (Volmer, 2005). Chlorpromazine given at 10 to 18 mg/kg IV was determined by Catravas *et al.* (1977) to prevent death in experimental dogs dosed with amphetamine when given early in the progression of clinical signs. Treatment decreased hyperthermia, convulsions, hypertension and heart rate. Normal respiration was maintained. Haloperidol at 1 mg/kg IV also decreased the clinical effects of amphetamines on body temperature, blood pressure, heart rate, respiration and decreased convulsions in experimental dogs. Chlorpromazine and haloperidol block  $\alpha$ -adrenergic and dopaminergic receptors. Short-acting barbiturates have also been recommended for treatment of amphetamine-induced seizures.

Cool IV fluids, ice packs, fans, cool water baths or cool moist towels, or gastric lavage with cool water can be used to treat hyperthermia. However, the shivering response may increase body temperature (Smith *et al.*, 2002).

Cardiac arrhythmias in the amphetamine overdose patient may resolve with treatment of central nervous signs (Smith *et al.*, 2002; Diniz *et al.*, 2003). Propranolol has been found to decrease the heart rate and blood pressure in experimental dogs given amphetamines, but did not improve survival (Catravas *et al.*, 1977). The use of  $\beta$ -blockers may lead to  $\alpha$ -receptor-mediated vasoconstriction and spasms of the coronary artery. Lidocaine, procainamide or amiodarone have been recommended to treat ventricular arrhythmias.

Urinary acidification with ascorbic acid or ammonium chloride increases amphetamine excretion but should not be attempted in the presence of acidosis or rhabdomyolysis, or if acid-base status cannot be monitored. Treatment of acidosis and rhabdomyolysis involves fluid diuresis and alkalinization. Respiratory support is sometimes required (Liechthi *et al.*, 2005). Treatment of hepatic damage with *N*-acetylcysteine has been recommended but the effectiveness is not known.

Urine or plasma samples may be tested for amphetamines at many diagnostic laboratories. Thin-layer chromatography is commonly used, and immunologic assays are available for some compounds. Gas chromatography and mass spectroscopy may be used for confirmation. There has been some success using over-the-counter drug test kits to detect amphetamines in canine urine (Teitler, 2009).

## Other stimulants

MDMA, sold as "ecstasy," is a common illegal club drug and will be discussed in some detail. Laws vary between

countries and states as to the legality of the herbal stimulant Khat and the related synthetic compounds mephedrone, and methylenedioxypyrovalerone, which have become common in the U.S. where they are sold in certain products with innocuous sounding names like "bath salts." Many legally available compounds are commonly used as stimulants. These include nicotine from tobacco products, methylxanthines such as caffeine, theobromine and theophylline found in common food and drink products, and alkaloids of the areca nut, present in betel quid. The latter compounds will be addressed briefly.

## MDMA

MDMA, more frequently known as "ecstasy," is a Schedule I drug. MDMA was used as an appetite suppressant and in psychotherapy (Smith *et al.*, 2002). The drug has shown some promise in the treatment of Parkinson's disease and post-traumatic stress disorder, but there are no accepted medical uses at this time (Morton, 2005). Currently, MDMA is one of the most popular club drugs in the U.S. and use increased 70% worldwide between 1995 and 2001 (Anonymous, 2011). A survey of U.S. students found that 5 to 6.7% of students have used MDMA in high school and 2% of 8th graders had used it (Eaton *et al.*, 2010; Latimer and Zur, 2010).

MDMA has been sold under numerous different names, a short list of which includes "Adam," "Batman," "bibs," "blue kisses," blue Nile," "charity," "clarity," "Debs," "decadence," "E," "Eve," "go," "happy pill," "hug drug," "lover's speed," "M," "roll" or "rolling," "Scooby snacks," "X" and "XTC." Tablets containing 50 to 150 mg MDMA are often brightly colored with pressed images such as product logos, butterflies, smiley faces, numbers or letters. Less commonly encountered are the powdered or capsule forms. "Ecstasy" tablets tested in Europe during the 1990s only contained 50% MDMA, on average (Libiseller *et al.*, 2005). Those tested in the U.S. averaged 83% MDMA (Anonymous, 2011). Other common ingredients included ketamine, methamphetamine, MDA and MDEA.

The LD<sub>50</sub> of MDMA in rats is between 160 and 325 mg/kg PO. Dogs given 15 mg/kg MDMA showed severe clinical signs and one of six of the dogs died (Frith *et al.*, 1987). MDMA is metabolized in the liver by N-demethylation to the active metabolite MDA (Smith *et al.*, 2002).

MDMA is structurally similar to amphetamines and mescaline, which may explain some of the clinical effects (Lyles and Cadet, 2003). MDMA increases neurotransmitter release, including serotonin, dopamine and NE (Smith *et al.*, 2002; Morton, 2005; Rimsza and Moses, 2005). Hallucinogenic effects have been attributed to serotonin release and inhibition of reuptake. This drug also inhibits MAO. MDMA directly binds to certain receptors, including 5-HT<sub>2</sub> receptors,  $\alpha_2$ -adrenergic receptors, M<sub>1</sub> muscarinic receptors and H<sub>1</sub> histamine

receptors, with less affinity for 5-HT<sub>1</sub>, dopamine, M<sub>1</sub>,  $\alpha_1$  and  $\beta$  receptors.

Frith *et al.* (1987) described the effects of MDMA given to dogs at increasing oral doses. Clinical signs began after about 45 minutes and continued for 6 to 8 hours. Dogs given low doses (3 mg/kg) most frequently showed signs of hyperactivity and mydriasis. Dogs given 9 mg/kg also became tachypneic with hypersalivation and circling behavior. One of six dogs given 15 mg/kg MDMA died after showing clinical signs that included vocalization, aggression, convulsions and front limb paralysis.

Onset and duration of clinical signs are similar in humans, though onset is more rapid with insufflation (Smith *et al.*, 2002; Rimsza and Moses, 2005). Mydriasis, delirium confusion, agitation, bruxism, tremors, seizures and loss of consciousness have been reported in MDMA users, as have hyperthermia and rhabdomyolysis (Kesseberth and Trammel, 1990; Smith *et al.*, 2002; Liechti *et al.*, 2005; Rimsza and Moses, 2005). Cardiovascular changes may include tachycardia or less commonly bradycardia, AV block and hypertension. A few deaths have been attributed to cardiopulmonary arrest (Liechti *et al.*, 2005) and liver failure (Andreu *et al.*, 1998; Gable, 2004; Rimsza and Moses, 2005; Liechti *et al.*, 2005). Onset of liver failure occurs days to weeks after exposure and the mechanism is not understood, though a hypersensitivity reaction has been suggested in some cases.

Treatment of MDMA exposure consists of appropriate gastrointestinal detoxification, as with amphetamines, and symptomatic and supportive care.

#### **Khat, mephedrone and methylenedioxypyrovalerone**

Khat (*Catha edulis*) is a plant found in East Africa and the Arabian Peninsula. Traditionally, leaves of this shrub have been chewed or consumed as a stimulant, but they are sometimes smoked. The leaves contain cathinone, a DEA Schedule I substance, which breaks down after about 36 hours, and the more stable compound cathine, which is a Schedule IV substance (Anonymous, 2011). Clinical signs associated with khat use include reduced appetite, tachycardia, hypertension and insomnia.

Mephedrone (4-methylmethcathinone) and methylenedioxypyrovalerone (MDPV) are compounds related to cathinone that have increased exponentially in popularity over the past few years. Mephedrone became illegal in the United Kingdom in April 2010, where it sells for approximately 20% of the cost of cocaine and is that nation's fourth most popular drug, after marijuana, cocaine and MDMA (Elwell, 2010). Mephedrone and MDPV are sold in the U.S. as "bath salts," under names such as "Ivory Snow," "Red Dove" and "Vanilla Sky."

Mephedrone and MDPV are snorted or ingested. Clinical signs usually last a few hours, but can last days (Elwell, 2010). Users report euphoria, increased energy

and become talkative. Adverse effects include epistaxis, mydriasis and cardiac arrhythmias. Later, users experience fatigue, dizziness and depression.

#### **Betel quid**

Though not common in most areas of the U.S., alkaloids of the areca nut are believed to be the fourth most commonly used psychoactive substance in the world after caffeine, ethanol and nicotine. It is estimated that 10 to 20% of the world population have access to "betel quid" containing these alkaloids, mostly in South and Southeast Asia and the Asia Pacific regions. These products can also be purchased in U.S. cities with large populations of Asian immigrants.

Palm trees of the genus *Areca* are cultivated in tropical and subtropical areas of the world to produce betel quid (Deng *et al.*, 2001). The nut of this palm tree may be ingested green, ripe and raw, baked, roasted, boiled, fermented or processed with various sweeteners. It is most commonly processed with leaves from the betel shrub, *Piper betle*, and calcium hydroxide. Tobacco and various spices are sometimes added. The betel quid industry is worth hundreds of millions of dollars. Betel quid is produced locally or under trade names such as Supari, Mainpuri tobacco, mawa, pan masala and Gutka. Gutka is illegal in some parts of India.

Alkaloids present in areca nut include arecoline, arecaine, guvacine and guvacoline. These alkaloids bind muscarinic receptors, induce coronary vasospasms and have weak activity on nicotinic receptors in ganglia.

Oral submucosal fibrosis is a preneoplastic disease that is common in young people who use betel quid. Clinical signs seen in inexperienced users and overdoses are more likely to be of interest to the veterinarian and may include mucosal irritation, nausea and vomiting, bronchoconstriction, hypersalivation, lacrimation, urinary incontinence, diarrhea, hypertension or hypotension, tachycardia, acute myocardial infarction and extrapyramidal signs. Most patients given supportive care recover within 24 h, and atropine has not been found to be effective as a treatment in humans (Deng *et al.*, 2001).

## **HALLUCINOGENS AND DISSOCIATIVE DRUGS**

The clinical effects of hallucinogenic and dissociative drugs are unpredictable and dependent very much on the individual and their environment (Nichols, 2004). Many compounds that alter consciousness have been termed "hallucinogens," though some, like marijuana and MDMA, only induce hallucinations under specific circumstances or at very high doses. "Psychomimetic"



is a term used to describe drugs that cause behavioral changes mimicking psychosis, but the value of this term in veterinary medicine is questionable. "Psychedelic" is a term commonly used by the media.

Lysergic acid diethylamide (LSD), a synthetic product similar to natural ergot alkaloids, is a common hallucinogen that acts on serotonin receptors. Hallucinogens with a similar mechanism of action include mescaline and N, N-dimethyltryptamine (DMT), described below. *Psilocybe* spp. and related mushroom species contain the hallucinogen psilocybin. Vomiting was reported after *Psilocybe* spp. mushroom ingestion in a dog (Spoerk, 2005). Behavioral changes in a horse suspected of ingesting *Psilocybe* spp. included fear and extreme aggression with recovery within 48 h (Hyde, 1990). Signs of severe toxicosis in a colt included hyperexcitability, tremors, mydriasis and bruxism. The colt was eventually euthanized due to traumatic injuries and weakness (Jones, 1990). Signs of psilocybin in cats are similar to those described below with LSD (Jacobs *et al.*, 1977). DMT is produced by several plants including *Psychotria viridis*, used to produce a tea known as Ayahuasca in the traditional practices of the indigenous people of the Amazon, *Anadenanthera peregrina* seeds, called yopo by native people of the Amazon rain forest, and *Phalaris* spp. of grass which grow around the world. Leaves of the plant *Salvia divinorum* are legal to possess and are commonly used to produce hallucinogenic effects, though by a unique mechanism described below.

Dissociative anesthetics are so called because they uncouple functions of the brain such as sensory and motor activity, emotion and consciousness, sometimes producing a cataleptic-type state (Branson, 2001). Affected individuals become disconnected from their environment and there is an absence of response to nociceptive stimuli. The synthetic drug phencyclidine is an example of a dissociative agent. Ketamine, a familiar therapeutic drug to most veterinarians, is a dissociative anesthetic and also a popular "club drug."

Several naturally occurring substances are used to produce hallucinogenic effects by various mechanisms. Certain species of mushrooms in the genus *Amanita*, specifically *A. muscaria* and *A. pantherina*, are sometimes intentionally ingested. These mushrooms contain ibotenic acid and muscimol, which bind glutamate receptors. Various plants containing atropine and scopolamine, including *Datura stramonium*, *Atropa belladonna*, *Mandragora officinarum* and *Hyoscyamus niger*, are routinely smoked or ingested (Halpern, 2004). The drug dextromethorphan is used recreationally for its dissociative effects.

## LSD and LSA

Lysergic diethyl amide (LSD) is the most powerful known hallucinogen (Nichols, 2004; O'Shea and Fagan, 2006). The d-isomer of LSD is responsible for the molecule's

effect on the CNS. The story of Albert Hoffman's synthesis and subsequent exposure to LSD is well documented elsewhere. LSD was marketed under the trade name Delysid and used in psychotherapy and for experimental purposes. Though there are no current medical uses for this drug, it has shown some promise for use in the treatment of alcoholism, drug addiction and obsessive-compulsive disorder. After becoming a popular recreational drug, LSD use was banned by the U.S. government in 1966, and it is currently a Schedule I drug (Nichols, 2004; Volmer, 2005).

LSD is a colorless, odorless and flavorless white powder that is usually dissolved in water, then applied to other substances such as blotter paper, microdots, tiny tablets, gelatin squares (termed "window pane" or "window glass"), stamps, gummy bears and other candies, and sugar cubes (Rimsza and Moses, 2005; Volmer, 2005; O'Shea and Fagan, 2006; Anonymous, 2011). Street names for LSD reflect these applications and include "acid," as in "spiked with acid," "blotter" or "blotter acid," "cubes," "dots" or "microdot," "L," "sugar" or "sugar cubes," "trip" or "wedding bells." Use of LSD is declining in the U.S. (Banken, 2004; Nichols, 2005). A recent survey found that 3% of high-school seniors had used LSD in the past 12 months (Latimer and Zur, 2011).

The seeds of *Ipomoea violacea*, the morning glory, contain lysergic acid amide (LSA), which is approximately 1/10th as potent as LSD, at a concentration of 0.02% dry matter (Halpern, 2004). Ingestion of 150 to 300 seeds causes clinical effects in humans (Frohne and Pfänder, 2004; Halpern, 2005; Volmer, 2005). The seed coat is protective, thus seeds must be crushed, germinated or soaked in water for ingestion to be effective. A word of caution to the adventurous: emetics are sometimes added to commercial morning glory seeds. Other sources of LSA include seeds of the Hawaiian baby woodrose, *Argyrea nervosa*, at a concentration of 0.14% dry matter, and endophyte-infected sleepy grass, *Stipa robusta*. *S. robusta* is present in the southwestern U.S.

## Toxicity

The effective dose of LSD for humans is between 0.05 and 0.20 mg. Products sold currently usually contain 0.04 to 0.06 mg and carry less likelihood of an adverse reaction than the pills sold in the 1960s, which could contain up to 0.25 mg LSD. Increasing the dose may produce both quantitative and qualitative differences in the response (Nichols, 2005). Some cats given intraperitoneal (IP) injections of 2.5 µg LSD/kg body weight showed mild clinical signs, and a dose of 50 µg/kg produced significant clinical signs in all cats tested (Jacobs *et al.*, 1972). The IV LD<sub>50</sub> for rats is 16 mg/kg (Volmer, 2005).

## Toxicokinetics

LSD is rapidly absorbed after ingestion in humans (Riordan *et al.*, 2002; Volmer, 2005). Peak plasma



concentrations occur within 6 hours and LSD is approximately 80% protein bound. Metabolism occurs primarily in the liver by hydroxylation and glucuronide conjugation to an inactive metabolite. A dose of LSD is 89% excreted in the feces and the elimination half-life is between 2 and 5h. Clinical effects can persist for 12 hours (Nichols, 2004).

### Mechanism of action

Many “recreational” hallucinogens act primarily as antagonists at serotonin receptors (Volmer, 2005; O’Shea and Fagan, 2006). LSD is structurally similar to serotonin. Actions at the 5-HT<sub>2A</sub> receptor are believed to be responsible for the hallucinogenic effects, though the signaling pathways involved have not been completely elucidated. Affected 5-HT<sub>2A</sub> receptors are located in the pyramidal cells of the prefrontal cortex, the reticular nucleus of the thalamus, and possibly the locus coeruleus, where the effect is to alter sensory processing. LSD and some other hallucinogens also have a strong affinity for the 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub> and other serotonin receptors, but the significance of this is not understood (Nichols, 2004). LSD causes increased release of glutamate in the prefrontal cortex, has a high affinity for dopamine receptors D<sub>1</sub> and D<sub>2</sub>, and shows some affinity for  $\alpha_1$  and  $\alpha_2$  adrenergic receptors (Nichols, 2004; Volmer, 2005).

### Clinical signs

General signs reported in nonhuman animals include disorientation, mydriasis, depression or excitation, and vocalization (Volmer, 2005). Behavioral changes reported in cats given IP injections of LSD included paw flicking, head and body shaking, yawning, chops licking, bizarre sitting and standing positions, falling from their perch, leaping about, compulsive scratching at the litter, pawing at the water, biting objects in the cage, play behaviors including pawing and sniffing objects, tail chasing and “hallucinatory behavior,” described as tracking, staring at, batting at, or pouncing on objects that were not apparent to the observer, frequent defecation and occasionally emesis. Grooming behaviors included rubbing the head with paws, licking, biting and scratching, and were sometimes “incomplete” – the cat would lick or bite the air or stick her tongue out. Rage behavior and vocalization were not seen in these cats (Jacobs *et al.*, 1977). Based on this study, one can only imagine the presenting complaint in a companion animal after accidental exposure to LSD.

### Treatment

Treatment is based on close observation with supportive and symptomatic care. Because absorption of LSD is relatively rapid and effects are self-limiting, gastrointestinal decontamination is unlikely to be beneficial

(Riordan *et al.*, 2002; Volmer, 2005). Clinical signs sometimes persist for up to 12h after ingestion of a large dose. Animals should be kept in a quiet, dark room to minimize sensory stimulation and restraint should be minimized to prevent hyperthermia. Laboratory techniques that have been employed in the detection of LSD include immunoassays, thin-layer chromatography, HPLC and LC/MS.

### Phencyclidine and ketamine

Phencyclidine, 1-(1-phenylcyclohexyl)piperidine, is a Schedule II synthetic drug with more than 80 known analogs. One such analog, ketamine or 2((-o-chlorophenyl)-2-methylamino)-cyclohexanone HCl, has less than 1/10th the potency of phencyclidine and is a Schedule III drug. Phencyclidine was originally used as an anesthetic under the trade names Sernyl for human patients and later Sernylan for veterinary patients. Sernyl, given IV, produces decreased response to nociceptive stimuli with insignificant respiratory and cardiac depression and no loss of corneal, papillary or other reflexes (Branson, 2001). However, Sernyl was associated with postoperative psychosis, dysphoria, delirium, violent behavior and hallucinations (Kisseberth and Trammel, 1990; Pestaner and Southall, 2003; Volmer, 2005; Anonymous, 2011). Sernylan was used in dogs, nonhuman primates, and was the preferred anesthetic for crocodilians (Ortega, 1967; Stunkard and Miller, 1974). Phencyclidine was last used commercially in the United States in 1978.

Phencyclidine is easily synthesized in clandestine laboratories. It is available in powdered or crystal form which may be dissolved in liquids such as water or “embalming fluid,” which contains formaldehyde and methanol, or it may be sold in tablets or capsules. Street products range from 5 to 90% purity and phencyclidine is sometimes substituted for THC, mescaline, LSD, amphetamine or cocaine. Phencyclidine is said to have a distinctive odor (Anonymous, 2011). This drug is frequently sold under the name “PCP” but other terms include “angel dust” or “angel hair,” “boat” or “love boat,” “dummy dust,” “CJ,” “hog” or “hog dust,” “PeaCe Pill,” “rocket fuel,” “stardust,” “whack” and “zombie dust.” “Embalming fluid” is added to cigarettes containing tobacco, marijuana or other leafy material such as parsley, mint or oregano. Names for this type of product include “supergrass,” “amp,” “happy sticks,” “sherm” and “wet sticks.”

Ketamine is not as readily produced by the amateur chemist but is used in veterinary and, less commonly, human medicine. Ketamine is labeled for use in cats and nonhuman primates. It is still used by physicians in pediatrics and in emergency and critical care situations where maintenance of blood pressure and respiration are crucial. Ketamine is an odorless, flavorless liquid which is often dried and crystallized then powdered for illicit use.

Ketamine can be ingested, injected, insufflated, placed into tobacco or marijuana cigarettes and smoked and has been used to "spike" drinks. Street names for ketamine include "cat valium," "green," "jet," "K," "special K," or "vitamin K," "keets," "kit-kat," "super acid" and "super C."

### Toxicity

Phencyclidine given orally at doses of 2.5 to 10 mg/kg produces clinical effects in dogs and doses of 25 mg/kg were lethal in all of six dogs treated (Kisseberth and Trammel, 1990; Volmer, 2005). Given via intramuscular (IM) injection, 2 mg/kg caused muscular incoordination, 5 mg/kg caused immobilization and convulsions in one of five dogs, and 15 mg/kg caused convulsions in both of two dogs treated (Ortega, 1967).

Ketamine has a high therapeutic index in domestic mammals. The sedative dose in cats is 20 mg/kg IM. Doses of 5 to 10 mg/kg IM have produced convulsive seizures in dogs. IV injections of 2 mg/kg produce ataxia in sheep and dissociative analgesia in cattle. Doses of 5 to 15 mg/kg IV caused struggling and vocalization in goats (Branson, 2001). The approximate LD<sub>50</sub> for ketamine in rodents is 600 mg/kg PO (Gable, 2004). Large doses of ketamine do not appear to produce analgesia in chickens or pigeons, but pigeons have gone into respiratory failure after being dosed with 0.11 mg/kg. A dose of 0.05 to 0.1 mg/kg produced anesthesia in parakeets, but 0.5 mg/kg IM was lethal (Branson, 2001).

### Toxicokinetics

Phencyclidine is ionized at gastric pH, thus little is absorbed in the stomach but there is significant intestinal absorption (Kisseberth and Trammel, 1990; Volmer, 2005). Inhaled phencyclidine is well absorbed. The drug is lipophilic, with a wide tissue distribution that includes the CNS, adipose tissue and gastric secretions. The latter leads to recycling and increases the biological half-life. Metabolism is variable between species. In the dog, approximately 68% of a given dose undergoes hepatic metabolism to a monohydroxyl form which is then conjugated for excretion. The remaining 32% is excreted unchanged in the urine. Approximately 88% of a dose of phencyclidine is excreted unchanged in the urine in cats.

Peak plasma concentrations in cats occur 10 minutes after IM injection of ketamine (Branson, 2001; Volmer, 2005). Ketamine is about 50% bound to plasma proteins in horses. It is distributed to the brain, adipose tissue, liver, lung and other tissues. Ketamine undergoes hepatic metabolism via N-demethylation or hydroxylation and glucuronide conjugation to a water soluble metabolite for excretion in the urine. The elimination half-life is approximately 67 minutes in the cat after parenteral dosing, 60 minutes in the calf, 42 minutes in the horse after IV dosing and 2 to 3 hours in the human (Branson, 2001).

### Mechanism of action

How compounds like phencyclidine and ketamine produce dissociative effects is not entirely understood. It is known that these drugs act on sites in the cerebral cortex, thalamus and limbic system (Branson, 2001; Pal *et al.*, 2002; Volmer, 2005). The effects on behavior and cognition in humans may mimic schizophrenia. Phencyclidine and ketamine bind to N-methyl-D-aspartate (NMDA) receptors at a different site than glutamate, the excitatory neurotransmitter, and therefore act as noncompetitive inhibitors. Reuptake of NE, dopamine and serotonin is decreased through inhibition of the biogenic amine reuptake complex. The sympathomimetic effects are associated with changes in heart rate, blood pressure and cardiac output and increased myocardial oxygen consumption. Phencyclidine inhibits  $\gamma$ -aminobutyric acid. Cardiovascular effects are more prominent with ketamine than phencyclidine.

Ketamine induces stage I and stage II anesthesia but not stage III. Ketamine acts on non-NMDA glutamate receptors, dopaminergic receptors, nicotinic receptors, muscarinic receptors and opioid receptors. Analgesic properties may be attributed to the actions of ketamine on opioid receptors. Ketamine binds dopamine receptors in dogs.

### Clinical signs

Dogs dosed with phencyclidine appear depressed at low doses and stimulated at high doses, with the potential for convulsive seizures (Branson, 2001). Onset of clinical effects is within 2 minutes of IV dosing (Ortega, 1967). Signs reported in dogs include muscular rigidity, grimacing facial expression, increased motor activity, head weaving, stereotyped sniffing behaviors, blank staring, incoordination, hypersalivation, nystagmus, opisthotonos, ascending loss of motor function, coma, tonic-clonic convulsions and hyperthermia (Ortega, 1967; Kisseberth and Trammel, 1990; Volmer, 2005). Cardiovascular effects include tachycardia, hypertension and cardiac arrhythmia. Deaths have been attributed to respiratory failure. Signs associated with phencyclidine in swine include muscle tremors and hypersalivation (Jones, 1972). Behavioral changes have been noted in neonates that were exposed during gestation (Branson, 2001). Reported changes in clinical chemistry parameters include acidosis, hypoglycemia, electrolyte imbalances and increased creatine phosphokinase and aspartate transaminase. Postmortem lesions associated with phencyclidine include epicardial and subendocardial hemorrhage and pulmonary congestion and hemorrhage (Kisseberth, 1990; Volmer, 2005).

Pineal, pedal, photic, corneal, papillary, laryngeal and pharyngeal, and other reflexes, are maintained during ketamine anesthesia (Branson, 2001; Volmer, 2005). There are significant species differences in the effects of ketamine. Ketamine increases muscle tone in cats, sometimes

causing forelimb extensor rigidity or opisthotonos. Other signs in cats include mydriasis and fixed staring. Hypersalivation and chops licking are associated with oral dosing. Most cats can sit within 2 hours and recover completely within 10 hours. Ketamine causes excitation in dogs and can produce tonic-clonic seizures. Pulmonary edema was reported in a dog 2 days after anesthesia with ketamine and xylazine. Hypersalivation, apneustic breathing and increased pulse are reported in sheep. Goats salivate, struggle and vocalize but usually recover within 20 minutes of dosing. Nonhuman primates have increased heart and respiratory rates and hypertension.

### Treatment

Prognosis for an animal overdosed with phencyclidine or ketamine is generally good with early intervention, but self-induced trauma and rhabdomyolysis complicate treatment. Cats that have undergone ketamine anesthesia usually recover rapidly and are able to sit up within 2 hours (Branson, 2001). Dogs injected with low doses of phencyclidine (1 mg/kg IM) recovered almost completely in a little over an hour, and at higher doses (5 mg/kg) were able to sit up within 2 hours (Ortega, 1967).

Treatment of phencyclidine or ketamine overdose is generally symptomatic and supportive. Animals must be kept in a dark, quiet room and restraint is to be avoided.

Inducing emesis is of limited use due to the rapid absorption of phencyclidine, but may be attempted in very recent ingestions of large doses if no contraindications exist (Kisseberth and Trammel, 1990). Activated charcoal binds phencyclidine, prevents recycling and is known to reduce mortality in dogs and rats. Repeated dosing of activated charcoal and cathartics is recommended due to enterohepatic cycling. Since these are lipophilic compounds, administering intravenous lipid emulsion may decrease the clinical effects.

Forced diuresis with mannitol or furosemide will increase the rate of clearance. Urinary acidification enhances excretion but is contraindicated in the presence of acidosis or rhabdomyolysis. Electrolyte abnormalities and hypoglycemia should be corrected as necessary.

Body temperature of the patient should be monitored. Cardiorespiratory abnormalities have been reported in some species. Muscle rigidity, hyperactivity and seizures associated with ketamine have been treated successfully with diazepam. Severe seizures require barbiturates or general anesthesia. Phenothiazine tranquilizers are believed to decrease the seizure threshold, exacerbate anticholinergic effects and produce hypotension.

### Other compounds

Mescaline, the active compound in peyote, is a well-studied hallucinogen due to its significance both

culturally and in the illicit drug trade. Salvinorin-A is an agent with hallucinogenic potential that is found in the legal herb *Salvia divinorum*.

### Mescaline

Use of mescaline (3,4,5-trimethoxyphenethylamine) dates back to approximately 8500 BC (Bruhn *et al.*, 2002). It is derived from several species of cactus including *Lophophora williamsii*, the peyote *Echinopsis pachanoi*, the San Pedro cactus, and *E. peruvianus*, the Peruvian torch cactus. The latter two cacti are native to South America and are common ornamental plants. Both can be boiled into a soup for ingestion. Peyote grows in the southwestern U.S. and Mexico and is used legally by indigenous people. A tuberous root grows into the ground and is capped by crowns or "buttons" which are removed. The buttons may contain 1.5% mescaline on a dry matter basis. Buttons can be eaten fresh but are often dried. Dried buttons are chewed, powdered, reconstituted or steeped into a tea. Pickled buttons have caused botulism (Halpern, 2004). Though traditional uses are permitted, mescaline is a Schedule I drug.

Mescaline is well absorbed by oral and parenteral routes in the dog, with maximum plasma concentrations detected within an hour of ingestion. Mescaline concentrations detected in the brain reflect blood concentrations. Renal, hepatic and splenic concentrations are three to six times blood concentrations. Mescaline can be detected in the urine within 30 minutes of exposure and remains detectable for up to 24 hours. Between 28 and 46% of a given dose is excreted unchanged in the urine (Chochin *et al.*, 1950). The mescaline molecule is structurally similar to serotonin and acts on the 5-HT<sub>2A</sub> receptor (Nichols, 2004).

Clinical signs of mescaline intoxication in dogs include vomiting, mydriasis, injection of the conjunctiva, hyperreflexia, chewing motions of the jaw and either excitation and disorientation or profound depression. Dogs are reported to recover from severe depression within 10 hours (Chochin *et al.*, 1950). Signs in humans are fairly similar and may include emesis, especially after ingestion of *Echinopsis* spp. (Halpern, 2004). Other signs that have been reported are mydriasis, sweating, hallucinations, synesthesia, disorientation, incoordination, increased heart rate and blood pressure, and hyperthermia.

### *Salvia divinorum*

*S. divinorum*, known as salvia, is a perennial sage closely related to mint. The plant is native to Mexico and grows in humid, semitropical climates. It is cultivated in California and Hawaii (Anonymous, 2011). The plant is of cultural significance to the Mazatec Indians of Oaxaca, Mexico, where it is used to treat diarrhea, headaches,



rheumatism and semi-magical diseases (Prisinzano, 2005). These indigenous Mexicans either chew the fresh leaves or extract juice. Salvia is also smoked (Frohne and Pfänder, 2005; Prisinzano, 2005). Salvia is a controlled substance in Denmark, Austria and Italy.

Salvia contains various diterpenes, including salvinorins A through F and divinorins A through C. Similar compounds are present in plants of the genus *Coleus*. Biological effects are attributed to salvinorin-A. Salvinorin-A is absorbed through the oral mucosa (Halpern, 2004). Little is known about the metabolism of this compound, but the elimination half-life in non-human primates averages 55.6 minutes (Prisinzano, 2005). Unlike other traditional hallucinogens, salvinorin-A is an agonist at the  $\kappa$  receptor and has no known effect on the 5-HT<sub>2A</sub> receptor. No toxic effects were seen in mice given high doses of salvia. No poisonings have been reported in animals.

## CONCLUSIONS

Toxicoses due to ingestion of illegal substances are infrequently reported in veterinary literature, but anecdotally common. With the high incidence of "recreational" drug use in the United States, occasional companion animal exposures are expected. Exposures are through accidental ingestion of the owner or neighbor's private "stash" or intentional exposure, either with lethal intent or as an attempt to get the pet "stoned." Though companion animals are far more likely to ingest "recreational" drugs, there are documented exposures of cattle and horses to illegally cultivated marijuana and illegal drugs have been used to alter the performance of horses.

There are a large variety of drugs used for recreational purposes, and their actions vary mechanistically, producing a broad range of clinical signs. Treatment requires detoxification and symptomatic and supportive therapy. Rarely are specific antidotes available, though antagonists are available for opioid drugs. Quantity of the drug ingested and severity of clinical signs are important factors for determining treatment regimen and prognosis. Some drugs, however, such as marijuana, can cause severe CNS signs in animals, but have an excellent prognosis with early and appropriate intervention.

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# Aluminum

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## INTRODUCTION

Aluminum (Al) is the third most abundant element that occurs naturally in the earth's crust. It is released to the environment both by natural processes and from anthropogenic sources. Small amounts of Al are released into the environment from coal-fired power plants, incinerators and roofing and siding materials. In addition, Al and Al-based compounds are used for a variety of purposes, from medicines (such as antacids, buffered analgesics, antidiarrheal agents and anti-ulcerative agents) to explosives and fireworks. Today, the use of Al is on the decline compared to two decades ago, but it is still used in a wide range of products and as a result poses risks to both animal and human health. Under some circumstances, exposure of animals and humans to Al is inevitable. Poisoning in animals by Al is rare, but in several incidents the outcome has been very serious or deadly. Among all Al compounds, Al phosphide is of major concern to animals, because at a low stomach pH, phosphide converts to toxic phosphine ( $\text{PH}_3$ ) gas. Al has been widely studied for its toxicological effects, especially for neurotoxicity, developmental and neurobehavioral toxicity in laboratory animals. This chapter focuses on Al toxicity in animals.

## BACKGROUND

Aluminum (Al) metal is obtained from Al-containing minerals, primarily bauxite. It is silvery-white in appearance and light in weight. Al is a component of beverage

cans, pots and pans, siding and roofing materials and foil. It is also found in products such as antacids, astringents, buffered aspirin, antiperspirants, toothpaste and food additives. In powdered form, Al is used in fireworks and explosives (ATSDR, 2006). Al occurs ubiquitously in the environment in the form of Al oxide, Al chlorhydrate, Al hydroxide, Al chloride, Al lactate, Al phosphate and Al nitrate. Because of a high reactivity Al does not exist as the metal in the environment, instead it combines with other elements such as sodium and fluorine and as complexes with organic matter.

Aluminum (Al) has a predilection for the brain and nervous tissues, therefore it touches every organ in the body via its nerve connections. The implication of Al in the etiology of a neurodegenerative disease like Alzheimer's has been well studied, and its effects at the nuclear, cytoplasmic, cytoskeletal, membrane, synaptic and neurotransmitter levels have been well studied (McLachlan, 1995; Savory *et al.*, 2006; Krewski *et al.*, 2007; Garcia *et al.*, 2010). Al exposure may also promote the onset of Parkinson's disease (Bondy, 2010). Its role has also been established in encephalopathy and amyotrophic sclerosis (Deloncle and Pages, 1997). In dogs, chronic Al exposure and its toxicity is linked to Cognitive Dysfunction Syndrome (CDS) or Doggie Alzheimer's Syndrome. Sometimes, a similar syndrome is observed in cats. These animals exhibit memory loss and personality change, and finally they fail to recognize their owners.

From a toxicological perspective, Al phosphide, which is commonly used as a pesticide, has been encountered in the poisoning of cows. In these cases, a majority of the toxicity comes from phosphine ( $\text{PH}_3$ ) gas which is generated from phosphide in the GI tract. Acute toxicity of

Al in animals is rare, but following subacute or chronic exposure, Al can produce a variety of toxicological effects. Frank *et al.* (1992) reported multifocal polioencephalomyelomalacia in Simmental calves associated with elevated Al levels. Adverse effects due to high Al levels have been observed in many domestic and wild-life species and fish (Allen, 1984; Sparling and Lowe, 1996). Al has also been involved in the etiology of grass tetany (Allen *et al.*, 1980). Since Al readily crosses the blood–brain barrier and the placental barrier, it appears that neurotoxicity and developmental toxicity are of particular concern in relation to Al toxicity (Gupta, 2009; Kumar and Gill, 2009; Domingo, 2011).

## TOXICOKINETICS

Aluminum (Al) is poorly absorbed following either oral or inhalation exposure, and practically none is absorbed following dermal exposure. In general, approximately 0.01–5% of ingested and 1.5–2% of an inhaled dose of Al is absorbed. Bioavailability of Al depends upon its chemical form and particle size (inhalation). For example, Al nitrate has shown to be twice as bioavailable as Al chloride in rats (Yokel and McNamara, 1988). In Wistar rats receiving a single gavage dose of Al hydroxide, Al citrate, Al citrate with added sodium citrate or Al maltonate, the fractional intestinal absorptions were 0.1, 0.7, 5.1 and 0.1%, respectively. In rabbits, following a single oral dose of the water soluble compounds, Al chloride (333mg Al/kg), Al nitrate (934mg Al/kg), Al citrate (1081mg Al/kg) and Al lactate (2942mg Al/kg), Al absorption was 0.57, 1.16, 2.18 and 6.3%, respectively (Yokel and McNamara, 1988). It appears that the oral absorption of Al can vary ten-fold based on chemical form alone, i.e., less absorption for water insoluble forms and more for water soluble forms.

Evidence suggests that following ingestion, Al is primarily absorbed in the duodenum and jejunum. The acidic pH of the stomach may solubilize Al from insoluble species such as  $\text{Al}(\text{OH})_3$ , facilitating absorption (Yokel, 1997). Furthermore, bioavailability can be influenced by other factors, such as variable amounts of essential and nonessential trace minerals, metal binding ligands and other dietary constituents, that can enhance or inhibit Al absorption. It is well established that concurrent consumption of  $\text{Al}(\text{OH})_3$  with fruit juices or with some common organic constituents of the diet (citrate, ascorbate, lactate, succinate, etc.) can markedly increase the absorption of Al.

The main mechanism of absorption of Al is probably passive diffusion through paracellular pathways. Another proposed mechanism of absorption for Al is an energy-dependent process that involves calcium

channels. Al may be taken up into mucosal cells, which may provide a barrier to its absorption. Small amounts of Al may then be slowly released into circulation (Van der Voet, 1992). Free Al ions occur in very low concentrations, because they complex with many molecules in the body, such as organic acids, amino acids, nucleotides, phosphates, carbohydrates and macromolecules. Therefore, toxicokinetics and toxicodynamics of Al can vary depending on the nature of these complexes.

The mean plasma half-life of Al after intravenous administration in dogs is approximately 4.5h. From the circulation, Al distributes to every organ, and the highest concentration is found in the bone. Lungs have the highest concentrations after inhalation. Approximately 50% of the Al body burden is in the skeleton and 25% is in the lungs. Long-term oral exposure to Al results in an increase in Al levels in the bone and from there it is slowly released (Ahn *et al.*, 1995; Krewski *et al.*, 2007). Al also accumulates in the brain, kidneys, liver and in hematopoietic tissue. Slow elimination coupled with continued exposure may produce an increasing body burden of Al. Furthermore, Al levels increase with age, especially in the lungs. Within cells, Al accumulates in the lysosome, cell nucleus and chromatin. In the blood, about 80–90% of Al binds to plasma proteins (Wilhelm *et al.*, 1990). Evidence suggests that Al primarily binds to transferrin, and small amounts to albumin. It is important to note that binding of Al to albumin is nonspecific and much weaker than to transferrin.

In general, brain Al concentrations are lower than in many other tissues. Al is known to cross the blood–brain barrier (BBB) and enter the brain by transferrin receptor-mediated endocytosis. Following inhalation exposure, Al can enter the brain by two mechanisms: (1) via the olfactory tract and (2) via nasal epithelium and axonal transport (Perl and Good, 1987; Zatta *et al.*, 1993). It is noteworthy that the cells which accumulate the most Al are long-lived postmitotic cells, such as neurons (Ganrot, 1986). In rabbits, increases of four- to ten-fold and ten- to 20-fold Al concentrations in brain are associated with neurotoxicity and death, respectively. Al is actively removed from the brain by means of an energy-dependent process.

Regardless of the route of exposure, Al is primarily excreted from the circulation into the urine and very little in the bile. Renal elimination of Al depends upon the Al complex. For example, Al bound in a low-molecular-weight complex could be filtered at the renal glomeruli and excreted, while Al in a high-molecular-weight complex would not. Animal studies suggest that following a single exposure, Al levels in urine can elevate as much as 14-fold. Al is primarily excreted in the urine during the first 24h period, and returns to normal levels 5 days post-exposure (Ittel *et al.*, 1987). The rate of Al clearance is consistent with glomerular filtration rate (GFR), although proximal tubular Al reabsorption and

Al excretion in the distal nephron have been suggested. Several animal studies have revealed a decrease in Al clearance and an increase in  $t_{1/2}$  with increased Al concentration. This may be due to the high Al concentrations that probably formed unfilterable Al complexes, thereby reducing the plasma filterable Al fraction. Because of the limited GI tract absorption of Al, only a limited amount of Al excretes in the milk. Following oral ingestion, unabsorbed Al excretes in the feces. For further details on toxicokinetics and toxicodynamics of Al, readers are referred to [Krewski \*et al.\* \(2007\)](#).

## MECHANISM OF ACTION

The central nervous system (CNS) and skeletal system appear to be the two major target organs for Al toxicity. It has been known for a while that Al is involved in neurodegenerative diseases like Alzheimer's, encephalopathy and amyotrophic sclerosis ([Deloncle and Pages, 1997](#); [Savory \*et al.\*, 2006](#); [Krewski \*et al.\*, 2007](#); [Garcia \*et al.\*, 2010](#); [Bondy, 2010](#)). However, the exact mechanism by which Al induces neurotoxicity is yet to be elucidated. Al mainly deposits in the hippocampus, cortex and amygdala, which are the areas of brain that are also rich in glutamatergic neurons as well as in transferrin receptors. Upon entering the brain, Al displaces physiological cations, such as magnesium, calcium or iron, and modulates their metabolism. Studies suggest that Al neurotoxicity can be modulated by the levels of tissue and cytoplasmic calcium. Al, by replacing calcium in the synaptic area, perturbs neurotransmitter release and alters neurotransmitter systems. High Al levels appear to modify cholinergic neurotransmission. Al is known to associate with many epithelia and endothelia, including the BBB, and may be responsible for compromising the properties and integrity of these membranes ([Wen and Wisniewski, 1985](#); [Exley, 1996](#)). It has been demonstrated that in some conditions, Al can cross the BBB without altering the functional characteristics of the membranes, while in other conditions Al interacts with the BBB with subsequent effects upon its barrier function ([Vorbodt \*et al.\*, 1994](#)). Al-related toxicological effects are noticed on both sides of the BBB ([Deloncle and Pages, 1997](#)). In neurons, glutamic acid in the form of a stable Al–glutamate complex is unable to detoxify cellular ammonia, which leads to neuronal death ([Harris, 1992](#)). Important events such as accumulation of Al in lysosomes (protease-rich vacuoles) and the hyperphosphorylation of neurofilaments (NFs) are involved in the molecular mechanism of Al-induced neurotoxicity ([Bizzi and Gambetti, 1986](#); [Ganrot, 1986](#); [Delamarche, 1993](#)). Recent findings suggest that oxidative and nitrosative stresses are involved

as mechanisms in Al-induced toxicity ([Kim, 2003](#); [Satoh \*et al.\*, 2005](#); [Garcia \*et al.\*, 2010](#)).

Al competes with magnesium in the biological system despite an oxidation state difference, and binds to transferrin and citrate in the blood stream ([Ganrot, 1986](#); [McDonald and Martin, 1988](#)). Al may also affect second messenger systems and calcium availability ([Birchall and Chappell, 1988](#)), and irreversibly bind to cell nucleus components ([Crapper-McLachlan, 1989](#); [Dryssen \*et al.\*, 1987](#)). In addition, Al has been shown to inhibit neuronal microtubule formation.

## TOXICITY

Acute poisoning of Al in animals is rare, but a high acute dose or repeated long-term exposure can lead to serious toxicological effects. Toxicity of Al depends on its chemical form, route of exposure and animal species. Oral LD<sub>50</sub> values for Al nitrate in Sprague-Dawley rats and Swiss Webster mice are reported to be 261 and 286 mg Al/kg, respectively ([Llobet \*et al.\*, 1987](#)). For Al bromide, these values are 162 and 164 mg Al/kg, respectively. LD<sub>50</sub> values for Al chloride in Sprague-Dawley rats, Swiss Webster mice and male Dobra Voda mice are 370, 222 and 770 mg Al/kg, respectively ([Ondreicka \*et al.\*, 1966](#); [Llobet \*et al.\*, 1987](#)). The LD<sub>50</sub> value for Al sulfate in male Dobra Voda mice is reported to be 980 mg Al/kg. A single gavage exposure to 540 mg/kg as Al lactate was found to be lethal in female New Zealand rabbits ([Yokel and McNamara, 1985](#)). In subchronic and chronic studies, Al has been found to cause lethality in mice ([Golub \*et al.\*, 1987](#)), but not in rats ([Dixon \*et al.\*, 1979](#)) and dogs ([Pettersen \*et al.\*, 1990](#)). In dogs, the signs of Al toxicity may include dermatitis, coryza and nasal discharges, loss of black pigment on the nose pad, and aggressive and violent behavior.

Toxic effects of Al depend on the target organ. Such effects may in part be related to Al deposition and substitution of physiological elements, such as calcium, magnesium and iron. Alterations by Al deposits can occur in: (1) the bone, interfering in heme synthesis leading to anemia, (2) the myocardium, leading to myocardial infarction and (3) the brain, leading to neurotoxicity and cognitive impairment. In addition, Al can cause hepatic and renal dysfunction and osteoarthritis.

There is conclusive evidence that Al compounds are neurotoxic to laboratory animals following an oral exposure. Marked signs of neurotoxicity, including ataxia, splaying and dragging of hind limbs, and paralysis occurred in maternal mice that were exposed during gestation and lactation to Al with estimated doses of 184 mg Al/kg/day ([Golub \*et al.\*, 1987](#)) or 250 mg Al/kg/day ([Golub \*et al.\*, 1992](#)) as Al lactate. Adult mice



that consumed 195mg Al/kg/day as Al chloride for 5–7 weeks in a diet that also contained 3.5% sodium citrate showed neurotoxic effects (Oteiza *et al.*, 1993). The citrate is likely to have enhanced the responses of Al neurotoxicity. Changes in brain biogenic amines (decreased dopamine and 5-hydroxytryptamine, and increased norepinephrine) occurred in rats that were treated with 21.4mg Al/kg/day as Al nitrate by gavage for 6 weeks (Flora *et al.*, 1991). Recently, Bondy (2010) also reported that acute exposure to Al can cause clinical neurotoxicity.

Evidence suggests that more serious disturbances occur in the brain following Al exposure. An alteration of the blood–brain barrier (BBB) would be necessary for Al brain transfer, but the mechanism leading to this alteration is still completely unknown, even though Al-induced peroxidation is perhaps a process involved therein (DeLoncle and Pages, 1997). Elevated Al levels have been related to impaired motor function and to a number of cognitive deficiencies in both humans and experimental animals (Sturman and Wisniewski, 1988). Neurofibrillar degeneration has been observed in brain regions that exhibit elevated Al levels (Krishnan *et al.*, 1988).

Histopathologic changes in the brain of rats receiving 92mg Al/kg/day as Al chloride and a high level of Al citrate (598mg/kg/day) for 6 months, showed extensive vacuolization in the astrocytes, swelling of astrocytic processes, neuronal vacuolization and nuclear inclusions (Florence *et al.*, 1994). In other studies, increased Al levels and histological alterations in the brain (particularly increased numbers of abnormal and damaged neurons and reductions in cell density in areas of the hippocampus and neocortex) also occurred in rats that received an estimated 12mg Al/kg/day as Al fluoride in drinking water and base diet for 45–52 weeks (Varner *et al.*, 1993, 1998).

There is some evidence that neurotoxic agents given prenatally induce subtle neurobehavioral impairment and delayed development of nervous system functions without any morphological malformation (Vorhees *et al.*, 1979; Adams and Buelke-Sam, 1981). The entry of Al into the developing nervous system is enhanced by the immaturity of the BBB (Thomas *et al.*, 1989), and the increased neuronal expression of transferrin receptors (Mollgard *et al.*, 1987). Al crosses the placental barrier, accumulates in fetal tissues and produces embryo/fetal toxicity, birth defects and developmental and neurobehavioral toxicity. Reproductive and developmental effects of Al have been discussed in detail in the “Placental Toxicity” chapter of this book and elsewhere (Golub and Domingo, 1998; Garcia *et al.*, 2011).

It is important to mention that the acute toxicity associated with Al phosphide ingestion is primarily due to the formation of the highly toxic gas phosphine (PH<sub>3</sub>), instead of Al. There are incidences in which cows died by ingesting grains treated with Al phosphide that was used for fumigation.

Chronic exposure to Al results in an increase in Al levels in bones, and this may result in bone abnormalities including reduced bone formation and demineralization, or even osteoarthritis. Osteomalacia is observed in dogs and pigs exposed to Al. Chronic inhalation exposure to Al can cause pulmonary fibrosis. In addition, long-term exposure to Al can cause hepatic and renal failure, and endocrine disruption. There is no evidence that Al is carcinogenic.

## Diagnosis

Diagnosis of Al poisoning can be based on a history of Al exposure, clinical signs and confirmation of Al in animal tissues. Using an Atomic absorption spectrometer or an inductively coupled plasma, Al can be measured in tissue, blood, urine, feces and hair. Only urine measurement can indicate whether recent exposure to excess levels of Al has occurred. High concentrations in the lung usually reflect inhalation exposure and elevated levels in bone, liver and spleen reflect sequestration (Yokel, 1977). In the diagnostic setting, liver and kidney are tested for Al and stomach/rumen content for phosphine (PH<sub>3</sub>) when aluminum phosphide poisoning is suspected. Aluminum levels in the range of 6–11ppm in the liver and 4–5ppm in the kidney of cows and sheep are regarded as toxic levels. In dog liver, an Al level >1.2 ppm is considered high (Puls, 1994). Aluminum levels greater than 1200 ppm in the diet are considered toxic to cattle and sheep. Presently, there are insufficient data to relate aluminum exposure levels with blood or urine levels.

## TREATMENT

There is no specific antidote for acute Al toxicity. So, treatment relies upon symptomatic and supportive therapies. Use of activated charcoal and cathartics can be rewarding. In the case of Al phosphide, sodium bicarbonate (5% solution) can be administered to stop conversion of phosphide to phosphine gas. There is evidence that dietary Silicone (Si) can reduce gastrointestinal Al absorption and increase its elimination. Dietary Si can also reduce brain Al accumulations. In the case of chronic exposure to Al, chelation therapy with deferoxamine or 3-hydroxypyridine-4-ones is very effective.

## CONCLUSIONS

Aluminum is a commonly occurring metal in the environment, and as a result a small amount is normally present in the body. A single exposure or repeat exposure

to higher levels of Al or its compounds (particularly Al phosphide) often leads to serious toxic effects, including neurotoxicity or death. Chronic low-dose exposure can cause neurodegenerative damage involving multiple mechanisms. Since Al crosses the BBB and placental barrier, it produces neurotoxicity, and reproductive/developmental and neurobehavioral toxicity. There is no specific antidote for acute Al toxicity. Chelating agents such as deferoxamine and 3-hydroxypyridine-4-ones can be effective in reducing Al body burdens.

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# Arsenic

Tam Garland

## INTRODUCTION

The ubiquitous element arsenic (As) is a nonmetal or metalloid in group V of the period chart. Frequently it is referred to as arsenic metal and is classified for many toxicological purposes as a metal. It exists in several forms and has a long history of various uses. It has been used in preparations from insecticides to wood preservatives, herbicides and even has some medicinal uses. It is responsible for many poisonings in people, and animals, both large and small.

## BACKGROUND

Arsenic is a ubiquitous element with several different forms. The form may determine the toxicity. The prevalent valences are the +3 and the +5 form. Arsenic is found in both an organic form and an inorganic form with valence numbers ranging from +3 to +5. As<sup>+3</sup> or arsenite is more toxic than arsenate, or As<sup>+5</sup>. The toxicity of arsenic is determined by its form (Vahter, 1993).

It is found as different ores and rocks, which are mined, then smelted resulting in elemental arsenic and arsenic trioxide. In the environment, arsenic usually exists as the pentavalent form and soil microorganisms may methylate it. Since it is ubiquitous in many forms it is not likely that complete avoidance is possible.

Arsenic's sources and uses have a long and varied history. A partial list is available in Table 31.1.

## PHARMACOKINETICS/ TOXICOKINETICS

Different toxic disease syndromes are caused by the different forms of arsenic. Inorganic arsenicals and trivalent organics cause a disease syndromes characterized by an effect on the gastrointestinal (GI) tract and the capillaries. In extremely low doses it is possible that the body will develop a tolerance to the arsenic. Pentavalent organic arsenicals produce a neurological syndrome.

There are many factors influencing the absorption of arsenicals. Among those variables are the form of the metal, the particle size, the purity, the solubility, the species affected and the physical condition of the animal exposed. Susceptibility to inorganic arsenicals varies among species, being highest in humans, followed by dogs, rats and mice (Harrison *et al.*, 1958, Hays, 1982). So clearly there are many variables affecting the absorption and toxicity of this metal, which increases the difficulty of making accurate predictions of lethal amounts.

Pentavalent organic arsenicals are better absorbed than are the trivalent arsenicals, especially through the GI tract. Small amounts of either form may be absorbed via the intact skin, but it usually remains locally within the skin. However, absorption is limited by the size of the arsenical particle size. If the particle size is too large, it is not absorbed. Hence, a more toxic arsenical that is not absorbed because of large particle size may effectively be less toxic.

Once arsenicals are absorbed, the distribution is through the blood to all the organs of the body. Arsenic



TABLE 31.1 Sources and uses of arsenic

Sources	Valence/form	Uses
Commercial uses and products	Inorganic arsenic trioxide (+3) Inorganic sodium arsenite (+3) Inorganic copper acetoarsenite (+3) Inorganic arsenic trioxide (+3) Inorganic sodium arsenate (+5) Inorganic chromated copper arsenate (+5) Inorganic lead arsenate (+5) Organic pentavalent (+5)	insecticide, cattle dip (0.18%) defoliant (highly toxic) Paris green – insecticide (emerald green) smelters herbicide wood preservative insecticide and medicinal monosodium methylarsenate (MSMA) and disodium methylarsenate (DSMA) (highly toxic to cattle) ant bait leaded gasoline
Natural sources		ores, minerals, volcanoes ground water and soil
Medicinals	Potassium arsenite (+3) Organic trivalent arsenical Organic pentavalent arsenical Organic trivalent arsenical Organic pentavalent arsenical	Fowler's solution tonic/conditioner thiacetarsamide – heartworm treatment in dogs tryparsamide – trypanosomiasis – old melarsoprol – trypanocidal arsenical feed additives (arsanilic acid, sodium arsanilate, 3-nitro, 4-hydroxyphenylarsonic acid)

accumulates in the liver and is slowly distributed to the other tissues. The spleen, kidneys and lungs are able to accumulate large amounts of arsenic. Arsenic has been shown to cross into the placental barrier, particularly in monkeys, hamsters and gerbils (Flora *et al.*, 2011). Chronic doses are stored in the bone, the skin, and other keratinized tissues, such as skin, hair, hooves and nails (Agency for Toxic Substances and Disease Registry, 2007).

The biotransformation of the arsenicals is poorly understood. There is some conversion from the +5 state to the +3 state, but the redox equilibrium favors the +3.

Methylation occurs by microorganism in the soil, but inorganic arsenicals are also methylated *in vivo*. The *in vivo* process may aid in the detoxification process. The kidneys may reduce a small amount of pentavalent arsenic to the more toxic trivalent form.

Arsenicals are excreted through many processes. In most species, between 40 and 70% of the absorbed amount of pentavalent arsenicals are excreted through the urine within 48 hours (Vahter, 1983). It may also be excreted in much smaller quantities through the sweat. Trivalent forms of arsenic are excreted more slowly and through the bile into the feces.

## MECHANISM OF ACTION

Arsenite (+3) reacts with sulfhydryl groups (–SH) of proteins and inhibits the enzymes by blocking the active groups. The arsenite inhibits alpha-keto oxidases which

contain dithiol groups and are involved in oxidation of pyruvate. Lipoic acid, an essential co-enzyme for pyruvic acid oxidase, and alpha-oxyglutaric acid oxidase are inhibited by the arsenite. These play an essential role in the tricarboxylic acid cycle. Actively dividing cells that have a high oxidative energy requirement are most susceptible to the effects of arsenicals.

Arsenites induce vasodilation and can cause capillary damage. The cellular integrity of the capillary is affected by an unknown mechanism. Evidence of vascular instability is seen by the presence of congestion, edema and hemorrhage in most of the visceral organs of animals with acute poisoning. This same mechanism of action occurs with inorganic arsenicals and with organic trivalent arsenicals, and they may be considered as “vascular poisons” (Hann and McHugo, 1960; Jubb and Huxtable, 1993; Agency for Toxic Substances and Disease Registry, 2007).

Arsenates (+5) are a little different. They are uncouplers of oxidative phosphorylation. The inorganic pentavalents may substitute phosphate in this reaction. The result is an increase in body temperature. Organic pentavalents have an unknown mechanism of action. There is some thought that they may interfere with vitamins B<sub>6</sub> and B<sub>12</sub>, which may allow for the demyelination and subsequent axonal degeneration that occurs.

Although arsenicals have been classified as carcinogens in people, this has not been the case in animals. Experimentally there have attempts to document arsenic-related cancer in animals but the experiments have been unsuccessful (Chan and Huff, 1997; Agency for Toxic Substances and Disease Registry, 2007; Hughes *et al.*, 2011).

## TOXICITY

Inorganic arsenicals are up to ten times more toxic than pentavalent arsenicals. The order of toxicity from greatest to least follows this schematic: inorganic  $\text{As}^{+3}$  (arsenite) > inorganic  $\text{As}^{+5}$  (arsenate) > trivalent organics > pentavalent organics ( $\text{I As}^{+3} > \text{I As}^{+5} > \text{O As}^{+3} > \text{O As}^{+5}$ ). In other metal toxicities, the organics are more toxic but with arsenicals the inorganics are the more toxic.

Toxicity is also influenced by many factors, including particle size. The more finely ground, the more surface area there is for reactions. Solutions, such as dips and defoliants, are the most dangerous. However, the causes of the poisonings are varied. Debilitated animals are more sensitive. Since arsenic is not biodegradable, the soil and the old corrals around old dipping vats are still sources for arsenic poisoning. The area around smelters is also a source of poisoning, similar to that of dipping vats. Human mistakes and carelessness are the largest contributing factors to toxic events. For example, feeding a product known as gin trash instead of cotton seed hulls has resulted in numerous animals being poisoned.

Clinical signs caused by either inorganic or trivalent aliphatic arsenicals are similar. Peracute toxicities often result in sudden death within minutes to a few hours, if the dose is high, of dissolved arsenic ingestion. Acute poisonings have more clinical signs: abdominal pain or colic, vomiting, a staggering gait and weakness, clear incoordination, rapid weak pulse and shock, diarrhea, followed collapse and death. If the acute poisoning is through dermal contact, then the arsenic will also be systemic. The skin will have blisters, edema and may be cracked and bleeding, leaving the skin susceptible for secondary infection (National Academy of Science, 1977; Evinger and Blakemaore, 1984). Those receiving a lower dose over a period of time may have subacute poisonings and will likely live several days, developing depression and anorexia. Movements may be difficult, stiff and incoordinated. Diarrhea is dark and possibly hemorrhagic and very fluid. Hematuria may be present, or the urine may contain protein and casts (National Academy of Science, 1977; Osweiler *et al.*, 1985). However, those suffering chronic poisoning are easily fatigued and have dyspnea when they are moved. These animals display intense thirst and have a rough dry hair coat as well as dry, brick-red mucous membranes. Cattle are described as having enlarged joints.

Clinical signs of phenylarsenic poisoning occur within 3 days of a high dose or after chronic exposure. Most noticeable are the neurological signs. The animal is generally bright and alert but uncoordinated. The animal may or may not be blind and these animals may have erythema in the skin. Some of the neurological damage may be reversible unless the nerves are damaged.

Lesions are often dependent upon the dose and survival times. There may be no lesions at all in animals that die from peracute poisoning. However, even these animals have some GI irritation. With the exception of peracute deaths, most of the other animals that die from some form of arsenic poisoning may have excess fluid in the GI tract. In cattle there is hyperemia of the abomasum and this may be the only finding. If there are other lesions in cattle, it is often necrosis of the rumen mucosal epithelium. Ruminants have gelatinous serosal edema in the rumen, reticulum, omasum and abomasum. The GI tract may have indications of irritation and be hemorrhagic. Lesions are indicative of capillary damage and the liver is usually soft and yellow.

The phenylarsonics (+5) are used in feed additives and lesions would be expected to be associated with overdoses in the feed mixture. A "downer pig" may have severe abrasions with muscle atrophy. Microscopic lesions indicate there is demyelination in the optic nerve and the posterior cord.

## TREATMENT

A diagnosis of arsenic poisoning is important and is based upon clinical history and signs. If more than one animal is involved, then lesions may also be important. Diagnostic arsenic levels in the kidney and liver are usually more than 8 to 10 parts per million (ppm), unless several days have lapsed since exposure, in which case it would likely be 2–4 ppm. Diagnostic levels of arsenic in the urine and feces are greater between 10 and 20 ppm. Arsenic should not be found in phenylarsonic acid intoxications.

Arsenic can poison large and small animals. If small animals are not showing clinical signs then evacuation of the stomach followed by lavage with 1% of sodium bicarbonate solution is recommended treatment. As a general rule early treatment, within 4 hours of exposure, is best. In carnivorous small animals, emetics followed by gastric lavage is the best treatment. The lavage may be milk and egg whites or with 1–5 grams of sodium thiosulfate. In herbivores treatment within 4 hours of exposure is also best. A large saline purgative, demulcents, 20 to 30 grams of sodium thiosulfate orally followed by a 10% solution three times a day for the next several days are also effective.

For both carnivores and herbivores, if treatment is more than 4 hours after exposure, then dimercaprol (British anti-lewisite, BAL) at 1.5 to 5mg/kg, intramuscularly, two to four times a day for 10 days or until recovery should be administered. Treatment should also include sodium thiosulfate at 30–40mg/kg administered

intravenously two to four times daily until recovery, which should be within 2 to 4 days.

If the diagnosis is a phenyl arsenic compound, there is no effective treatment for nerve damage caused by these compounds. BAL is not effective for these intoxications.

Alternative experimental treatments include DMPS (2,3-dimercaptopropanesulfonic acid, Dimerval) and DMSA (2-3, dimercatosuccinic acid or succimer). DMPS has been shown experimentally to chelate arsenic in dogs when dosed at 3–5mg/kg in very slow infusion over 20 minutes or 4–8mg/kg orally every 6 hours. Arsenic intoxication has also been experimentally treated with DMSA or succimer in dogs at 7.5mg/kg every 6 hours.

## CONCLUSIONS

Determining the diagnosis and initiating treatment early will determine the prognosis. Acutely poisoned animals have a poor prognosis without early intervention. Understanding the differentials is important to institute the appropriate treatment for the appropriate condition. Arsenic produces signs of severe gastroenteritis, similar to those of pancreatitis, viral or bacterial gastroenteritis, irritating plants, caustic agents and zinc phosphide poisoning. Likewise, other heavy metals will produce similar clinical signs. However, poisoning with phenylarsonics, used most frequently in feed additives, has a high morbidity rate but is associated with a low mortality rate. Recovery generally requires 2 to 4 weeks.

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# Cadmium

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## INTRODUCTION

Cadmium accumulation in plants and animals is increasing from several sources of environmental exposure. The application of rock phosphate (which contains varying amounts of cadmium depending on the source) and sewage sludge fertilizers results in cadmium deposition in the soil of pastures (Piscator, 1985; ATSDR, 2009). In addition to direct ingestion of soil containing cadmium, some forage plants extract cadmium from the soil. Although cadmium accumulation in the soft tissues of livestock has been demonstrated and there is ample experimental documentation of the toxicity of cadmium in animals, under natural conditions, documented cases of direct toxic or carcinogenic effects of cadmium in livestock have been uncommon (Dorn, 1979).

## BACKGROUND

Pure cadmium is a soft, silver-white metal with an atomic number of 48 and a molecular weight of 112.41. It is a divalent transition metal with chemical properties that are similar to zinc and is usually found as a mineral in combination with other elements to form cadmium oxide, cadmium chloride or cadmium sulfate (ATSDR, 2009). Numerous compounds are formed from cadmium and thus it is used in batteries, solders, semiconductors, solar cells, plastics stabilizers, and to plate iron and steel. All soil and rocks contain some cadmium. It can enter the environment from zinc smelting and refining, coal combustion, mine wastes, iron and steel production, and

from the use of rock phosphate and sewage sludge as fertilizers (Klasing, 2005).

Cadmium accumulation in plants and animals is increasing from a variety of sources, being the most severe in the vicinity of zinc smelters. The use of cadmium-containing mineral supplements in feed (e.g., from calcium phosphate), the application on pastures and hay fields of phosphate fertilizers (which contain varying amounts of cadmium depending on the source) and sewage sludge results in cadmium deposition in the soil (Piscator, 1985). Some plants readily extract cadmium from the soil thereby making it available for consumption. For example, cadmium concentrations in clover grown in soil fertilized with high cadmium rock phosphate were significantly higher than the concentrations in clover grown in soils treated with low cadmium phosphate fertilizer (McLaughlin *et al.*, 1997). A New Zealand national survey of soils and plants, and random testing of kidneys from grazing animals, revealed that there was an approximately two-fold increase in soil cadmium, while over a 3-year period, 14–20% of cattle kidneys exceeded the New Zealand maximum residue level of 1 µgCd/g (Roberts *et al.*, 1994). In a study where cattle were allowed to graze pastures treated with anaerobically digested sewage sludge for up to 8 years, cadmium was the only metal to accumulate consistently in increased amounts in the tissues of the cattle (Fitzgerald *et al.*, 1985). It has been reported that cattle grazing on sewage sludge-treated pastures consumed significantly more (up to three times) cadmium than cattle on control pastures (Reddy and Dorn, 1985). In addition, a Swedish study has found a direct correlation between cadmium in feed and pig kidneys (Grawe *et al.*, 1997). A study reporting the analysis of Wisconsin dairy feeds for heavy metals



found that cadmium concentrations in complete dairy feed rations were the closest of the heavy metals to U.S. maximum acceptable concentrations, suggesting that cadmium has the greatest potential to exceed those maximum standards if the amounts of cadmium in feeds increase in the future (Li *et al.*, 2005). However, several studies have failed to demonstrate any adverse clinical manifestations related to increased cadmium concentrations in the animals examined. In one study, although cattle on pasture fertilized with sewage sludge consumed increased amounts of cadmium and had increased fecal excretion and kidney accumulation of cadmium (Reddy *et al.*, 1985), there were no adverse health effects noted in these cattle (Dorn *et al.*, 1985). Similarly, when corn silage or corn that was grown on sewage sludge fertilized fields were fed to sheep or pigs, respectively, significant increases in kidney cadmium concentrations were measured, but no other adverse treatment-related effects were noted (Lisk *et al.*, 1982; Telford *et al.*, 1982). Although cadmium is of concern in the environment, and cattle grazing on cadmium-contaminated pastures have increased tissue concentrations of cadmium, two additional studies conclude that accumulation of cadmium in the liver and kidneys of cattle may be a moderately effective screen for the entry of cadmium into the human food chain, as long as liver and especially kidneys are not consumed (Sharma and Street, 1980; Johnson *et al.*, 1981). It has been reported that regardless of the concentrations of cadmium fed to livestock, the amount in meat, milk and eggs is always lower than that in the diet that the animal was eating. Thus, foods derived from those products decrease human exposure (Klasing, 2005). This is fortunate as chronic cadmium poisoning has been documented in humans. In these cases, it has been associated with osteoporosis, renal lesions, tissue mineral imbalances and death. In addition, the Department of Health and Human Services has determined that cadmium and cadmium compounds may be reasonably anticipated to be carcinogens.

### PHARMACOKINETICS/ TOXICOKINETICS

In animals, cadmium exposure is primarily through oral ingestion. Compared to other divalent cations such as zinc and iron, intestinal absorption of cadmium is relatively low, ranging from approximately 1 to 5% in most species, with up to as much as 16% in cattle, dependent on the dose (Klasing, 2005). Interestingly, cadmium bound to metallothionein in foods of animal origin is absorbed less efficiently than cadmium salts and therefore may be less available for uptake (Groten *et al.*, 1990).

After absorption, cadmium is transported in the plasma bound to albumin and in lesser amounts to other serum proteins. It distributes throughout the body with the highest concentrations in the liver and kidneys, which account for approximately one-half of the total cadmium in the body. Muscle and bone do not accumulate high concentrations of cadmium. Blood cadmium concentrations are indicators of recent exposure while urine cadmium is a better indicator of the body burden. Cadmium is not transported well into milk or eggs, or across the placental barrier (Klasing, 2005). In pregnant and lactating livestock, the toxicokinetics of cadmium have been compared. In this study, the kinetics of cadmium were measured in lactating versus non-lactating ewes after a single intravenous or oral administration of cadmium chloride. The non-lactating ewes exhibited a low cadmium bioavailability (0.12–0.22%), a large steady-state volume of distribution ( $23.8 \pm 5.41$ /kg), and a low blood clearance ( $0.20 \pm 0.031$ /kg/day) with a mean residence time of  $113 \pm 28$  days. The lactating ewes had a higher bioavailability (0.33–1.7%), and the mean residence time was close to that of the non-lactating ewes despite a greater blood clearance ( $0.46 \pm 0.0131$ /kg/day) because the volume of distribution of cadmium in the body was larger. The cadmium clearance in milk remained low in the lactating ewes (Houpert *et al.*, 1997).

In the body, cadmium is excreted very slowly, with daily losses of approximately 0.009% of the total via the urine and approximately 0.007% in the feces via the bile. Cadmium–protein complexes are excreted in the kidneys and then resorbed from the filtrate in the proximal tubules. This area of the renal cortex accumulates cadmium and is susceptible to damage and necrosis. Depending on the species, the biological half-life of cadmium can vary from months to years, which results in cadmium accumulating in animals as they age (Klasing, 2005). For example, several studies have documented age-related increases in cadmium in the kidneys of horses (Elinder *et al.*, 1981a; Anke *et al.*, 1989).

In mammals and birds, cadmium accumulates in the liver and kidneys at concentrations of 0.1–2.0 and 1–10 mg/kg wet weight, respectively. It has been discovered that animals with long life spans, such as horses, can accumulate large amounts of cadmium in their organs, particularly in their kidneys. In samples of renal cortex from old horses, concentrations of up to 200 mg/kg have been reported (Elinder, 1992).

### MECHANISM OF ACTION

Experimentally, acute exposure to high doses of inorganic cadmium leads to its accumulation in many organs,

eliciting liver and in some cases testicular damage (Dixit *et al.*, 1975; Habeebu *et al.*, 1998; Klasing, 2005). Once inside the cell, free cadmium binds to protein sulfhydryl groups, disrupting the cellular redox cycle, depleting glutathione and eliciting intracellular oxidant damage. In addition, its similarity to other divalent cations such as calcium interferes with their normal functioning (Klasing, 2005). Cadmium ions can displace zinc and other divalent metals from their binding sites on metalloproteins. For example, in the testis, cadmium can interfere with zinc proteins, leading to widespread apoptosis and necrosis (Xu *et al.*, 1999). In the liver, acute cadmium toxicity results in widespread hepatocyte apoptosis, followed by varying degrees of necrosis depending on the dose (Habeebu *et al.*, 1998). This is related, in part, to the effects of resident liver macrophages (Kupffer cells) to potentiate and increase the initial liver damage caused by cadmium alone. This has been demonstrated in several systems in which inhibition of Kupffer cells significantly decreases liver damage caused by a toxic dose of cadmium (Sauer *et al.*, 1997a, b).

Cadmium readily binds to, and induces the production of, metallothionein, a cysteine-rich, metal-binding protein. Binding to metallothionein does not have a major effect on the uptake of cadmium, but is, in part, responsible for retention of cadmium within cells and its long half-life (greater than 10 years in humans). Metallothionein does this by decreasing cadmium elimination, especially in bile. Within hepatocytes, metallothionein binds to cadmium, decreasing its hepatotoxicity. Experimentally, rats that have greater induction of metallothionein in the liver are somewhat protected from cadmium hepatotoxicity (Kuester *et al.*, 2002). However, in the kidneys the cadmium-metallothionein complex is nephrotoxic and it has been theorized that it may play a role in chronic poisoning in humans (Klaassen and Liu, 1997).

## TOXICITY

Increased exposure to cadmium in combination with zinc, lead and/or other metals continues to occur in the vicinity of non-ferrous metal smelters and processing facilities. These exposures have resulted in toxicoses, although it can be difficult to separate the effects of cadmium from those of lead, zinc and other metals. In one such case in the Netherlands, kidney cadmium concentrations were found to be twice those of cattle in control areas. However, although hemoglobin, blood iron concentrations and iron-binding capacity were lower in the cadmium-exposed cattle compared to controls, no adverse clinical effects were observed (Wentink *et al.*, 1992).

In an additional study in the Netherlands, bulls fed diets containing increased concentrations of cadmium, lead, mercury and arsenic had increased concentrations of cadmium in the kidney and liver, but did not exhibit histological lesions related to the intake of heavy metals (Vreman *et al.*, 1988). However, more recently, deaths in horses exposed to cadmium, lead and zinc from a non-ferrous metal processing plant in Eastern Europe were attributed to ingestion of these metals in their feed. Analysis of tissues from a number of these horses revealed extremely high concentrations of cadmium (40–100 times normal) and 3–6 times the normal concentrations of lead (Bianu and Nica, 2004). Toxicoses have also been reported in sheep and horses in the vicinity of non-ferrous metal smelters in China. Analysis of the tissues from these animals revealed lead and cadmium concentrations significantly higher than those of controls (Liu, 2003).

A survey of cadmium concentrations in tissues from healthy swine, cattle, dogs and horses in the midwestern United States was conducted in the mid-1970s. While the median cadmium concentration was low, at or below 0.6 ppm in the kidneys of cattle, swine and dogs, the median concentration in the kidneys was four times greater in horses (Penumarthi *et al.*, 1980). One study has indicated that horses may be more at risk for cadmium toxicity than other species. In this Swedish study, the cadmium concentrations in the kidney cortices of 69 otherwise normal horses were measured and correlated to any histological lesions that were noted. In that study, renal cadmium concentrations ranged from 11 to 186 µgCd/g wet weight, with an average of 60 µgCd/g. This study found a correlation between increased chronic interstitial nephritis and increasing cadmium concentrations in the renal cortex. There was no obvious relationship between the age and the frequency of renal lesions (Elinder *et al.*, 1981a). These same authors also found that cadmium concentrations in the kidney cortices were approximately 15 times greater than those in the liver of the same animals (Elinder *et al.*, 1981b). Age-dependent increases in kidney metallothionein and cadmium have also been reported in horses (Elinder *et al.*, 1981a; Jeffrey *et al.*, 1989; Plumlee *et al.*, 1996). However, these later studies and others (Holterman *et al.*, 1984) have not reported renal lesions similar to those reported by Elinder *et al.* (1981a). One diagnostic investigation has reported lameness and swollen joints, i.e., lesions of osteochondrosis, in addition to osteoporosis and nephrocalcinosis in horses near a zinc smelter in Pennsylvania. In the horses examined, kidney zinc and cadmium concentrations were elevated. In this case, it was postulated that the osteoporosis that was observed in one foal and the nephrocalcinosis seen in the foal and its dam were related to the elevated renal cadmium (Gunson *et al.*, 1982). When ponies were raised near a similar zinc smelter for periods of time up to 18.5 months, there were significant elevations in tissue zinc and cadmium concentrations.

Increases in tissue cadmium concentrations were correlated with increasing age, although increases in tissue zinc concentrations were not. Generalized osteochondrosis was present in joints of the limbs and cervical vertebrae, and there was lymphoid hyperplasia. From this study, it was concluded that the development of osteochondrosis was associated with increased exposure to zinc and possibly cadmium. However, other lesions of cadmium toxicosis, such as renal damage or osteomalacia, were not present (Kowalczyk *et al.*, 1986).

In wildlife, white-tailed deer (*Odocoileus virginianus*) harvested within 20 km of zinc smelters in Pennsylvania had very high kidney concentrations of cadmium and zinc. These deer were also reported to have had joint lesions similar to zinc-poisoned horses from the same area (Sileo and Beyer, 1985).

In humans, occupational exposure to cadmium has been associated with renal dysfunction and osteomalacia with osteoporosis. One of the earliest effects of chronic cadmium exposure is renal tubular damage with proteinuria (Bernard *et al.*, 1992). Other chronic effects can include liver damage, emphysema (through inhalation), osteomalacia, neurological impairment, testicular, pancreatic and adrenal damage, and anemia. Reproductive, developmental and tumorigenic effects have been reported in experimental animals (Lee and White, 1980; Thompson and Bannigan, 2008; Siu *et al.*, 2009; Flora *et al.*, 2011). Historically in the 1940s, high environmental exposure in one area of Japan from eating cadmium-contaminated rice resulted in itai-itai (ouch-ouch) disease. This was manifested by intense bone pains and pathological bone fractures, mainly in elderly women, with osteoporosis and renal dysfunction (Kobayashi, 1978). In addition, studies in Europe and China have demonstrated that low to moderate exposure to cadmium from zinc smelters resulted in a decrease in bone density, and an increase in fractures in women. In one of these studies, cadmium concentrations in the blood and urine were taken as biomarkers of exposure (Katzantzis, 2004). Experimental studies in animals have confirmed the adverse effects of cadmium on bones. Six mechanisms have been theorized to explain these effects: (1) interference with parathyroid hormone stimulation of vitamin D production in the kidney, (2) reduced renal vitamin D activation, (3) increased urinary excretion of calcium, (4) reduced intestinal calcium absorption, (5) interference with calcium deposition in bones and (6) interference with bone collagen production (Kjellstrom, 1992).

## TREATMENT

In animals, cadmium toxicosis is prevented by minimizing exposure in the environment and in feedstuffs.

## CONCLUDING REMARKS/FUTURE DIRECTIONS

Although toxicoses in domestic animals are uncommon, exposure is gradually increasing especially in grazing animals. Since chronic cadmium toxicity is of concern to humans, continued close observation and analysis of cadmium in animals is indicated to provide a system for environmental cadmium surveillance.

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## Chromium, iodine and phosphorus

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### CHROMIUM

Chromium is a metallic element that can exist in six valence states, with the trivalent chromium form most commonly found in nature as ferrochromite ores. Both trivalent and hexavalent (+6) are widely used in various industrial and manufacturing processes. Chromium is an essential trace element and functions in a number of metabolic processes including glucose, lipid and amino acid metabolism. Hexavalent chromium is considered to be more toxic than the trivalent form which may be a direct result of its increased systemic availability.

While hexavalent chromium is considered to be a human carcinogen, acute chromium toxicosis in animals is probably of minimum concern in all but the most unusual circumstances. Both hexavalent chromium and dichromate are easily converted to trivalent chromium in mammalian systems but the burning of chromium-treated lumber does not produce enough heat to convert the dichromate to the trivalent chromium form. Oil field contamination with hexavalent chromium has been associated with cases of cattle death (Thompson *et al.*, 1991) and a solution of strong, oxidizing chromium was responsible for dairy cattle deaths when the solution dripped on cattle and was absorbed dermally (Talcott *et al.*, 2005). Acute chromium toxicosis is associated with severe congestion and inflammation of the digestive tract, kidney damage and liver damage. Chronic toxicosis has been associated with gastroenteritis and dermatitis.

several common other forms including iodide ( $I^{-1}$ ) and iodate ( $IO^{-3}$ ). Iodine is widely distributed in nature in both organic and inorganic forms but only in low concentrations. Iodine is essential for the normal synthesis of thyroid hormones and a deficiency of iodine can result in thyroid enlargement or goiter. Deficiencies may occur from eating feeds grown on iodine-deficient soils or from the presence of goitrogenic substances (NRC, 2005a). High dietary iodine for a prolonged period of time can reduce the iodine uptake by the thyroid, causing the clinical syndrome of iodine deficiency.

Common dietary sources of iodine include iodized salt, ethylenediamine dihydroiodide (EDDI) and calcium iodate. Iodized salt can contain 0.01% iodine (100 ppm) and has not been associated with excess iodine exposure. EDDI has been used to prevent and treat foot rot in cattle, although its efficacy is unclear (Morgan, 2004). Toxic effects of iodine excess have been reported in cattle consuming iodine-containing feed additives (Thompson *et al.*, 1991). Clinical signs include decreased feed intake, decreased milk production, rapid breathing, nasal and ocular discharge, dry hair coat and non-responsive hock lesions. Affected cattle were on high intakes of iodine (68–600 mg/head/day and above) for several weeks before obvious clinical signs appeared. Young calves exhibited chronic coughing and profuse nasal discharge at 100–200 ppm dietary iodine. Removal of the excess iodine from the diet and supportive care usually results in rapid return to normal.

### IODINE

Iodine is a non-metallic element of the halogen group that occurs as a purple-black crystalline solid but has

### PHOSPHORUS

Most phosphorus in nature exists in combination with oxygen in the form of phosphates, primarily in igneous

and sedimentary rocks. Inorganic phosphates are commonly used as chemical fertilizers, food and feed supplements and have many industrial uses. Phosphorus is abundant in the animal body primarily as a structural component of crystalline hydroxyapatite in bone and teeth but also as required components of phospholipids, nucleic acids, nucleotides and enzyme cofactors. Phosphate ions also function in acid–base balance and other essential body functions. Phosphorus is an essential macroelement in nutrition and is an important consideration in the formulation of animal diets. The largest dietary source of phosphate will be in the form of inorganic phosphate supplements and other dietary sources may include plant-origin feeds as well as bone, meat, poultry and fish meals. Normal phosphorus nutrition and metabolism requires adequate calcium in the diet with an appropriate calcium-to-phosphorus ratio (Ca:P). While adverse effects of excess phosphorus are rare, they can occur with either excess dietary phosphates or deficient dietary calcium. If the Ca:P ratio is balanced, usually no wider than 2:1, animals can tolerate a wide range of dietary phosphorus levels (NRC, 2005b).

Excess phosphorus in the diet of ruminants, especially sheep, can result in the formation of urinary calculi in the kidney or bladder. This formation of stones can obstruct or completely block urine flow, especially in males, resulting in the bladder filling with urine and eventually rupturing into the abdominal cavity causing death. The problem can be prevented by correctly balancing calcium and phosphorus in the diet. Excess phosphorus in the diet of horses has resulted in nutritional secondary hyperparathyroidism, a condition usually associated with a high grain diet without appropriate calcium supplementation. The high dietary phosphate will depress the intestinal absorption of calcium with a decrease in plasma calcium and an increase in plasma phosphate levels. Low plasma calcium will stimulate the secretion of parathyroid hormone which will

increase bone mineral resorption activity. The skeletal bones will lose calcium and the demineralized bone will be replaced by fibrous connective tissue with the facial bones often becoming enlarged (Joyce *et al.*, 1971), leading to the common term of big head disease in horses. It is also known as bran disease, since feeding high dietary levels of bran, which is high in phosphate and low in calcium, has historically been a cause of the disease. In all animals, optimum animal performance will be closely associated with optimum dietary calcium and phosphorus balance.

Phosphorus, in the form of white or yellow phosphorus, has historically been used as a rodenticide but is uncommon today. Initial clinical signs following ingestion would include gastroenteritis with vomiting and diarrhea. If the animal survived several days it would often develop a secondary phase of severe liver damage with renal insult also occurring.

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# Copper

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## INTRODUCTION

Copper is a transition group metal with high electrical and thermal conductivity. The chemical abbreviation for copper is Cu, which comes from the Latin word *cuprum*, which refers to the island of Cyprus, known in the ancient times for large deposits of copper ore. The Bronze Age followed the discovery that adding tin (and other metals) to copper made the resulting metal alloy much more durable. Copper has been shown to be an essential element for both animals and plants but can be toxic under certain conditions. This chapter describes the toxicity and deficiency of copper in animals.

## BACKGROUND

Toxic insults from copper occur in two main categories, acute and chronic. The acute overexposure of animals to copper can occur by both oral and parenteral routes (Galey *et al.*, 1991) but is relatively uncommon. Sheep are very susceptible to chronic copper poisoning, where even a slight excess of copper in the diet can build up over weeks or months to cause problems. Cattle and other ruminants are less likely to suffer from chronic mild to moderate copper excesses in the diet but can be affected by relatively high levels (Gummow, 1996). Monogastric animals seem to tolerate excess dietary copper much better than ruminants and high dietary copper additions (up to 250 ppm) have been used for

growth promotant effects, such as in pigs and poultry. Finally, certain breeds of dogs (e.g., Bedlington terriers) have a genetic predisposition for liver copper accumulation similar to Wilson's disease in humans (Taboada and Thompson, 1997). Each of these may have a different clinical presentation with the main target organs for acute copper exposure generally being the gastrointestinal tract, liver and kidney with the target organs for chronic problems being the liver, red blood cells and kidney.

Dietary sources of copper include normal plant concentrations of copper, with forages and cereal grains usually containing less than 10 ppm copper on a dry matter (DM) basis. Some oilseed meals can contain up to 35 ppm copper in DM. By far the largest source of copper in the diet will be feed additives such as copper sulfate, copper chloride and copper oxide. Additional sources of copper for the animal may be chelated copper sources in the diet or the administration of boluses containing copper metal wires or other solid forms of copper that remain in the rumen/reticulum and slowly dissolve over time. Misformulation of rations or errors in the mixing of feed can result in high concentrations of copper. Problems can arise from the use of feed formulated for a copper-tolerant species when used for a copper-sensitive species such as sheep, or the use of trace mineralized salt in sheep diets. Other sources for large acute oral copper exposures would include copper sulfate foot baths and copper algacides or fungicides. Most water sources have low copper concentrations but the use of copper piping with slightly acidic water can result in additional copper being dissolved.

## PHARMACOKINETICS

Copper is primarily absorbed in the small intestine and transported in the blood by transcuprein and albumin, which serve to reduce the oxidative effects of divalent copper. In the liver, copper can be stored in the lysosomes, excreted in the bile or incorporated into ceruloplasmin for use and transport to cells in other parts of the body. Biliary excretion is the major mechanism responsible for copper homeostasis and identified genetic disorders of copper accumulation are due to impaired biliary excretion. Copper is utilized by essentially every cell in the body and there are several important copper-dependent enzymes including cytochrome C oxidase, superoxide dismutase, lysyl oxidase and dopamine beta hydroxylase.

In the ruminant, copper has a complex interrelationship with dietary molybdenum and sulfur which, when present in excess, will both decrease copper absorption and inhibit copper utilization. In the reducing environment of the rumen, excess sulfur favors the formation of sulfides which can inhibit copper absorption from the gastrointestinal tract. Additionally, the formation of tri- and tetrathiomolybdates can also inhibit the absorption of copper or, if in high amounts, can cause systemic effects by holding copper in a non-biologically available form (NRC, 2005).

## MECHANISM OF ACTION

Acute exposure to excess copper causes gastrointestinal irritation and can cause erosions of the mucosa as well as a blue-green discoloration of the contents and wall. Normally, the free copper concentration in cells is kept very low by copper-binding proteins such as metallothionein, glutathione and copper chaperone proteins. An excess of copper can overwhelm these binding proteins and allow free copper ions to exist in the cell which can directly bind proteins and nucleic acids. Additionally, the free copper can form reactive oxygen species and hydroxyl radicals, causing lipid peroxidation of membranes and damage to nucleic acids and cellular proteins (NRC, 2005).

Chronic copper toxicosis in sheep is caused by the inability of the sheep to increase the biliary excretion of copper in response to dietary increases. Copper will then accumulate in the liver but during this accumulation phase there will be little or no evidence of negative effects (Bremner, 1998). If accumulation continues, the liver can suffer direct damage from the high concentrations of copper. When damage is severe, hepatic necrosis develops and copper is released into the bloodstream. Additionally, stress to the animal can also accentuate this mobilization of copper from the liver and into the

bloodstream. Increasing copper concentrations in the bloodstream will overwhelm the protective transport actions of transcuprein and albumin, which can then result in the lysis of red blood cells due to the oxidation of the red blood cell membrane by ionic copper. As copper is mobilized from the liver, it can accumulate in the kidney. The kidney can be damaged both from the accumulation of copper as well as the direct toxic effects of hemoglobin following the hemolytic event. Cattle can also be affected by chronic copper toxicosis, with a strong suspicion of breed differences (Du *et al.*, 1996) but with hemolysis occurring with less frequency than in sheep.

Chronic copper toxicosis in dogs is primarily seen in the Bedlington terrier where genetic studies have shown it to be an autosomal recessive disorder (Forman *et al.*, 2005). Other breeds including West Highland White terriers, Skye terriers and Doberman pinschers (Speeti *et al.*, 1998) have breed-related hepatic copper accumulation, the origin of which is less defined. Chronic copper toxicosis in dogs will also have a period of copper accumulation without clinical signs or detectable damage to the liver. As copper concentrations increase, the animal develops a chronic active hepatitis with necrosis and inflammation. As with sheep, excess free copper damages many cellular components of the liver, including the lipid peroxidation of mitochondrial membranes. A sudden release of copper with the resulting hemolytic crisis is much less likely to occur in dogs. The disease usually appears at 2–6 years of age, with animals often showing no problems before this time.

## TOXICITY

There is a paucity of information on the amount of copper needed for acute poisoning in various species, but the general range is given as 25–50 mg/kg of body weight. Copper sulfate is most often implicated in clinical cases as the copper source. The toxic dose of copper sulfate in cattle is 200–800 mg/kg, with sheep more sensitive at 20–100 mg/kg. Affected animals show clinical signs of salivation, gastroenteritis and abdominal pain which may rapidly develop to dehydration, shock and death. Animals that survive longer than 24–48 h develop liver and kidney damage and the animal may have an acute hemolytic crisis. Postmortem findings include gastroenteritis and a blue-green discoloration to the gastrointestinal tract and contents. Varying degrees of liver and kidney lesions are present, with milder lesions in acute deaths and more dramatic lesions developing after 48 h. Diagnostic testing of the intestinal tract contents shows a high level of copper. In acute deaths, there are normal levels of copper in the liver and kidney. If the



animal survives over 24h, elevated copper levels are found in liver and kidney.

In sheep, even normal levels of copper in feeds (10–20ppm) can cause hepatic copper accumulation when the molybdenum level in feed is low (less than 1ppm). As the copper-to-molybdenum ratio (Cu:Mo) increases above 6:1, the risk of copper accumulation increases, with those diets whose ratio is above 20:1 being very dangerous for sheep. At the higher Cu:Mo ratios, toxic accumulation of copper in the liver can occur over a matter of weeks. In affected animals, there may be an acute hemolytic crisis and the animals show clinical signs of weakness, anorexia, icterus, dyspnea and pale mucous membranes. There may be hemoglobinuria and death is common among severely affected animals. Postmortem findings include icterus, swollen liver, enlarged spleen and the kidneys appear dark, often referred to as gunmetal blue or black kidneys. Antemortem testing of serum copper often shows an elevation above normal, but this decreases over time with a rapid decrease following fluid therapy. Postmortem diagnostic testing for copper should be performed on both liver and kidney. Mobilization of copper from the liver may reduce copper concentrations to normal levels but the mobilized copper is then accumulated in the kidney. The ratio should be tested for both copper and molybdenum. The close environment of the sheep should be inspected for extraneous sources of copper.

Young dogs with genetic susceptibility to chronic copper toxicosis may develop an acute syndrome of weakness, vomiting and anorexia. Older dogs may present with a more chronic syndrome of weight loss and anorexia, which may progress to ascites and neurological signs related to a developing hepatic encephalopathy. If untreated, the animal may succumb to liver dysfunction and postmortem findings may include a cirrhotic liver. Antemortem diagnostics would include an evaluation of liver enzymes. In suspect cases, a liver biopsy should be used to confirm elevated copper content (Taboada and Thompson, 1997).

## TREATMENT

Treatment of animals acutely poisoned with copper mainly consists of supportive treatment directed at the shock, dehydration and damage to the gastrointestinal tract.

Treatment of sheep with severe clinical signs following hemolytic crisis is often unrewarding. Supportive care should include fluid therapy and the consideration of a blood transfusion. Ammonium or sodium molybdate (50–500mg) and sodium thiosulfate (0.3–1g) should be used daily as a drench for up to 3 weeks not only in affected animals but also in other animals that have received the same diet. Ammonium tetrathiomolybdate has been suggested as a treatment but is difficult to

obtain. It can be administered IV or SQ at 1.7–3.4mg/kg on alternate days for three treatments. Molybdenum in the diet can be increased to 5ppm and zinc can be supplemented at 100ppm to reduce copper absorption.

Dogs affected with chronic copper toxicosis should be fed a low copper diet, e.g., avoiding organ meats which are usually higher in copper. The use of oral chelating agents is suggested to enhance urinary excretion of copper. The use of d-penicillamine at 10–15mg/kg PO twice daily or the use of trientine hydrochloride at 10–15mg/kg PO twice daily have been suggested. Liver enzymes should be monitored every 6 months and consideration be given to liver biopsy to assess liver copper concentrations. The addition of elemental zinc to the diet (100–200mg/day as the acetate) should be considered to reduce copper absorption.

## CONCLUSION

In trace amounts, copper is an essential element, but in excess it is a toxicant. In general, sheep and certain breeds of dogs are more susceptible to copper poisoning. Copper produces toxicity by multiple mechanisms. Poisoned animals are usually treated with ammonium molybdate and sodium thiosulfate. In addition, supportive care including fluid therapy and blood transfusion are beneficial.

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# Fluoride

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## INTRODUCTION

Fluorine is a member of the halogen group on the periodic table and is rarely found in elemental form in nature but instead exists as fluoride, the monovalent anion, combined with other elements. The most common mineral containing fluoride is fluorspar, also known as fluorite ( $\text{CaF}_2$ ), and soils generally contain calcium fluoride ( $\text{CaF}_2$ ). Although now rare, sodium fluoride and sodium fluorosilicate ( $\text{Na}_2\text{SiF}_6$ ) had been used as insecticides and anthelmintics. Sodium fluoroacetate (compound 1080) is another formerly used rodenticide that is rarely seen in the United States today but may be found in other parts of the world (e.g., Australia). Fluoroacetate can also be found naturally in several species of plants (*Gastrolobium* spp., *Oxylobium* spp. and others). Sodium fluoride, sodium fluorosilicate and fluorosilicic acid have been used in the United States for the fluoridation of drinking water for humans to prevent the development of dental caries (CDC, 2001). When the gas hydrogen fluoride (HF) is dissolved in water it forms hydrofluoric acid, a very hazardous chemical that has industrial and laboratory uses such as purifying metals, etching glass and cleaning semiconductors due to its ability to dissolve silicates and metal oxides. This chapter will stress the chronic effects of fluoride excess, also called fluorosis or fluoride toxicosis, in large animals.

## BACKGROUND

Fluoride is present at low levels in virtually all feed and water sources, thus animals will have continuing

exposure throughout their lifetime. While a small amount of fluoride in the diet has been shown to improve bone and teeth development, a chronic excess of fluoride can have adverse effects on teeth, bone and other body systems. Large animals have been exposed to excess fluorides through the ingestion of high-fluoride rock phosphates used as nutritional supplements, by the ingestion of forages contaminated with excess fluorides from industrial pollutants or volcanic emissions, or through water containing excess fluorides from industrial pollution or dissolved from natural sources (Shupe and Olson, 1971, 1983). Rock phosphates destined for animal diets must be defluorinated before use or have a phosphorus to fluorine ratio (P:F) of more than 100:1 in order to avoid exposing the animal to excess fluoride (Osweiler, 2004). Following a long history of problems, industrial contamination of forages and water with excess fluoride has decreased due to recognition of the problems caused by excess fluoride and increased regulatory controls on fluoride-emitting industries. Historical point sources for fluoride emissions have included the smelting industries (e.g., aluminum, copper and steel), brick or ceramic product factories, coal-fired power plants and the phosphate-processing industries. Plant uptake of fluoride by translocation from the soil is usually not an important source of fluoride for grazing animals. Much more significant sources include airborne fluoride that settles on plant surfaces and fluoride from soil that is ingested directly or contaminates the plant (NRC, 2005). Acute fluoride poisoning in large animals is rare but can occur following exposure to fluoride-containing commercial products (Bischoff *et al.*, 1999) or to ash and tephra following volcanic eruptions (Shanks, 1997).

## PHARMACOKINETICS AND MECHANISM OF ACTION

Sodium fluoride is readily absorbed from the digestive tract and is several times more biologically available than fluoride compounds from feed or environmental sources. Fluoride is distributed to all parts of the body with approximately 50% of absorbed fluoride being excreted by the kidneys. The remainder will be incorporated into bone and teeth with very little accumulation in the soft tissue. Fluoride concentrations in the blood, urine and soft tissues may reflect recent ingestions but will also increase slowly over time with continuing excess fluoride exposure and accumulation in the bone. Greater than 95% of the body burden of fluoride will be contained in the bones with bone levels dependent upon the amount of fluoride ingested, duration of exposure, bioavailability, species, age and diet of the animal involved. If dietary fluoride exposure decreases, bone fluoride levels will decrease slowly over a long period of time. In cattle there appears to be a partial placental barrier to the movement of fluoride to the fetus as even high levels of fluoride in the diet of the dam did not adversely affect the health of the calves, even though higher fetal blood and bone fluoride concentrations resulted (NRC, 2005). Fluoride is excreted in the milk but this does not appear to be a significant source for the neonate.

The major adverse effects of chronic excess fluoride ingestion concern the teeth and bones of affected animals. Fluoride substitutes for hydroxyl groups in the hydroxyapatite of the bone matrix which alters the mineralization and crystal structure of the bone. Bone changes induced by excess fluoride ingestion, termed skeletal fluorosis or osteofluorosis, include the interference of the normal sequences of osteogenesis and bone remodeling with the resulting production of abnormal bone or the resorption of normal bone. The fluoride content of bone can increase over a period of time without other noticeable changes in the bone structure or function. Once lesions start to develop, they are usually bilateral and symmetric. The most consistent gross changes are abnormal bone formation on the periosteal surface with thickening of the cortex. In cattle, earliest clinical changes usually occur on the ribs and mandible as well as the medial surfaces of the metatarsal and metacarpal bones. Histologically, bones will have abnormal remodeling and mineralization with irregular collagenous fibers and excess osteoid tissues. While an excess of ingested fluorides can adversely affect the bones at any time in the animal's life, the bones in younger animals are more responsive to the excess fluoride.

Dental fluorosis develops when the period of excess fluoride intake occurs during the period of tooth development; in cattle this will generally be before 30–36

months of age. Teeth are affected during development with damage to ameloblasts and odontoblasts and the resulting abnormal matrix unable to mineralize normally (Shearer *et al.*, 1978). Both enamel and dentine are adversely affected. Affected teeth may erupt with mottling (alternating white opaque horizontal areas or striations in the enamel), hypoplasia, dysplasia (abnormal soft dull white chalky enamel or horizontal zones of constriction), erosion or pitting of enamel and affected teeth are prone to excessive abrasion and discoloration.

Acute fluoride toxicosis occurs when soluble forms of fluoride (e.g., sodium fluoride) are ingested in large doses. Absorption is rapid and clinical signs can appear within 30–60 min following ingestion. Although the exact mechanism of action is not known with certainty, fluoride concentrations in blood and soft tissues rapidly increase which leads to hypocalcemia. Sudden death from acute fluoride exposure is thought to involve the development of hyperkalemia or diminished  $\text{Na}^+/\text{K}^+$ -ATPase activity and the inhibition of glycolysis (NRC, 2005). Fluoride can induce oxidative stress and modulate intracellular redox homeostasis, lipid peroxidation and protein carbonyl content. Fluoride is thought to inhibit the activity of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase. Depletion of glutathione results in excessive production of reactive oxygen species (ROS) at the mitochondrial level, leading to the damage of cellular components. Fluoride can also alter gene expression and cause apoptosis (Barbier *et al.*, 2010). Genes modulated by fluoride include those related to the stress response, metabolic enzymes, the cell cycle, cell–cell communication and signal transduction.

## TOXICITY

There are a number of factors that influence the amount of fluoride required to produce specific lesions and clinical signs including the amount of fluoride ingested, duration of exposure, bioavailability, species, age and diet of the animal involved. The point where fluoride ingestion becomes detrimental to the animal also varies from animal to animal. Clinical signs develop slowly and can be confused with other chronic problems. Animals often show non-specific intermittent stiffness and lameness, which appear to be associated with periosteal overgrowth leading to spurring and bridging near joints as well as ossification of ligaments, tendon sheaths and tendons. The clinical presentation may easily be confused with other conditions, such as degenerative arthritis, but the lesions associated with fluorosis are not primarily associated with articular surfaces. In severe cases,

affected cattle may become progressively lammer and eventually may refuse to stand or may stand with rear legs upright and be on their knees to graze (Shupe and Olson, 1983). Lameness in cattle leads to abnormal hoof wear with elongated toes, especially in the rear legs. In long-term studies with cattle on varying levels of fluoride intake, skeletal neoplasms were not seen even in cattle with severe osteofluoritic lesions (Shupe *et al.*, 1992).

A great deal of effort has gone into the classification of dental lesions in cattle produced by excess fluoride ingestion. The incisor teeth are evaluated for enamel defects and abrasion pattern. The usual classification system ranges from a value of 0 for normal teeth to a value of 5 for severe fluoride effects (Shearer *et al.*, 1978; Shupe *et al.*, 1992). Because of the nature and complexity of the disease these dental lesion should not be the sole criterion for diagnosis. In general, severely affected teeth appear with brown or black discoloration, may have enamel defects and show increased wear including exposure of the pulp cavity, which causes pain while chewing roughage or swallowing extremely cold water. There will be a correlation between lesions on incisor teeth and those cheek teeth that form and mineralize at the same time. Cheek teeth that are abnormally worn cause improper mastication with roughage being difficult for the animal to utilize. The animal will have variable and decreased intake and the decreased production, slowed growth and general poor health associated with poor nutritional status. Animals with chronic exposure to excess fluorides have dry skin and hair coat.

Acute fluoride poisoning of cattle can result in clinical signs of depression, weakness and ataxia with postmortem findings of gastroenteritis (Bischoff *et al.*, 1999) and degenerative changes in the renal tubular epithelium.

## TREATMENT

Diagnosis of chronic fluoride toxicosis is based upon clinical signs, exposure history, dental lesions, evaluation of lameness and bony lesions and elevated urinary fluoride concentrations. Normal cattle urine contains less than 6 ppm fluoride. Animals having recent exposure or continuing release from fluorotic bone will have 15–20 ppm urinary fluoride (Oswailer, 2004). The biopsy of a rib or coccygeal vertebrae for fluoride analysis, as well as radiographic examination of teeth or bones, may also be helpful. A full postmortem examination should be performed, with attention to bone and teeth for both gross and histopathologic examination. Various bone samples should be submitted for fluoride analysis including metatarsal, metacarpal, rib, pelvis and mandible. Normal values for cattle are 400–1200 ppm fluoride

on a dry, fat-free basis. Animals affected with chronic fluorosis can contain 3000–5000 ppm fluoride on a dry, fat-free basis. Analysis of feed, water or suspect material should also be included.

There is no specific antidote or treatment for chronic fluoride toxicosis. Sources of excess fluoride should be identified and removed from the diet. With reduction of dietary fluoride to background levels, mild to moderate bone changes may be reduced and normal bone laid down. Extensive bone lesions will not be remodeled to normal and teeth lesions are irreversible. Symptomatic and supportive care for animals with bone and teeth changes include providing high-quality easily masticated feeds, limited grazing area and provisions to avoid cold or frozen water. For mild fluorotic changes, improving the diet and grazing may avoid excess wear on the teeth and reduce mastication problems.

## CONCLUDING REMARKS

Animals can be intoxicated with fluoride from its higher concentrations in supplemented feed or drinking water. The level of fluoride contamination in drinking water is dependent on the nature of the rocks and the occurrence of fluoride-bearing minerals in ground water. While in small amounts fluoride is necessary for teeth and bone development, in higher concentrations fluoride is toxic. Fluoride causes toxicity through multiple mechanisms. In addition to inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase, fluoride can induce oxidative stress and modulate intracellular redox homeostasis, lipid peroxidation and protein carbonyl content, as well as alter gene expression and cause apoptosis. A chronic excess to fluoride can have adverse effects on teeth, bones and other body systems. Diagnosis is based on analysis of feed, water and bones. There is no specific treatment for fluoride toxicosis.

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# Iron

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## INTRODUCTION

Iron is an essential element necessary for animal and plant life. It works as an oxygen (O<sub>2</sub>) carrier in hemoglobin/myoglobin and is involved in numerous biological oxidation–reduction reactions, including photosynthesis. Iron is present in cytochrome P450 and is crucial for the metabolism of many chemicals in the liver, kidney and other organs. Deficiencies in iron can result in anemia. Excess iron can cause iron overload and organ damage, while oxidation of ferrous to ferric iron in hemoglobin results in methemoglobinemia and an inability of red blood cells (RBCs) to carry O<sub>2</sub>. Consequently, because of its importance, the uptake, distribution, storage and excretion of iron is very tightly regulated in animals under normal conditions. Because of its reactivity, free iron compounds are sequestered with proteins to ensure that they do not initiate intracellular oxidative damage through electron donation and the formation of reactive oxygen species (ROS) such as hydroxyl radical. In this chapter, we will deal primarily with clinical animal exposures to excess iron through ingestion, parenteral administration or genetic iron storage abnormalities. Nutritional deficiencies or exposure to chemicals causing methemoglobin formation such as nitrates in ruminants, or nitrites and chlorates in all species, are discussed elsewhere.

## BACKGROUND

Iron is very abundant in the universe and is the fourth most abundant element on earth. Its atomic number is 26

and its atomic weight is 55.847. It has been recognized to be an essential nutrient for more than 100 years and is present in all the cells of the body. The largest amount of iron is incorporated into the proteins, hemoglobin and myoglobin. Within RBCs (erythrocytes), hemoglobin transports O<sub>2</sub> from the lungs to cells throughout the body, while myoglobin binds O<sub>2</sub> for use in muscle cells (Klasing, 2005). Iron present in the serum is bound to the protein transferrin, and in milk is bound to lactoferrin. Iron-containing proteins in the mitochondrial electron transport chain are essential for oxidative phosphorylation and energy production. Iron is also contained in enzymes of the Krebs cycle and in cytochromes P450 which are necessary for the metabolism of chemicals (Fairbanks, 1994).

The iron content of feedstuffs can be highly variable depending on the components. Iron in plants is subject to wide variation depending on the type of plant and the amount of iron in the soil, while many animal-based feed components are often rich in usable iron. The iron content of water can also vary greatly (Klasing, 2005). Large amounts of iron in water give the water a rusty color and a metallic taste, but upper limits of iron in drinking water for livestock and poultry have not been established since experimental data are not sufficient to make definite recommendations (NRC, 1974).

Worldwide, iron-deficiency anemias affect large numbers of people, but nutritional iron deficiencies are much less of a problem in animals. However, there are groups of animals which are vulnerable to iron deficiency including newborn piglets, veal calves and those animals with parasitic infestations (Underwood, 1977). For instance, newborn piglets have very low concentrations of liver iron (29mg/kg) compared to newborn rabbits whose liver iron stores average 135mg/kg.

There is accumulating experimental evidence in laboratory animals that excessive iron deposits in the brain, and alterations in iron metabolism, play an important role in neurodegenerative diseases (Connor *et al.*, 1995; Lan and Jiang, 1997; Fredriksson *et al.*, 1999; Dal-Pizzol *et al.*, 2001; Qian and Shen, 2001; Arosio and Levi, 2002; Jomova *et al.*, 2010).

## PHARMACOKINETICS/ TOXICOKINETICS

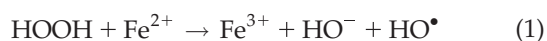
The homeostatic regulation of iron in the body is complex and involves uptake, transport, utilization, storage and loss. Under normal conditions, iron is poorly absorbed from most diets with approximately 5–15% absorbed from the gastrointestinal (GI) tract. This uptake can double in iron deficiency. The body has a very limited ability to excrete iron, therefore iron homeostasis is maintained by adjusting iron absorption to the body's needs. The amount of dietary iron that is absorbed through the GI tract is determined by the needs of the individual animal and is inversely related to serum ferritin concentrations (Bothwell *et al.*, 1979). There are four main factors influencing iron absorption in the GI tract: (1) individual factors including the animal's age, iron status, and health, (2) conditions in the GI tract, (3) the chemical form and amount of iron ingested and (4) other components of the diet which can enhance or reduce intestinal absorption. Iron is absorbed by enterocytes of the small intestine in ferrous ( $\text{Fe}^{2+}$ ) form and transferred to the serum where it is converted to the ferric ( $\text{Fe}^{3+}$ ) form and bound to transferrin (Goyer and Clarkson, 2001). In normal animals, the majority of fecal iron comes from ingested iron which is not absorbed. Once absorbed, the body vigorously retains ingested iron unless bleeding occurs with daily iron loss limited to about 0.01% (of the body total) per day (Goyer and Clarkson, 2001). It has been found that even in the face of hemolytic anemia with destruction of erythrocytes, less than 1% of the iron is excreted in the urine and feces (Underwood, 1977).

In the bloodstream, serum iron is primarily bound to transferrin with lesser amounts bound to ferritin. Iron in the serum forms a pool from which it enters, is transported, leaves and re-enters at a variable rate for the synthesis of hemoglobin, ferritin, cytochromes and other iron-containing proteins. Of the total iron in the body, approximately two-thirds is bound to hemoglobin and 10% to myoglobin and iron-containing enzymes, with the remainder bound to the storage proteins ferritin and hemosiderin (Goyer and Clarkson, 2001). Ferritin and hemosiderin are found throughout the body with the main concentrations being in the liver, spleen and bone marrow. They are protective in that they keep cellular

iron in a bound form. Ferritin contains up to 20% iron while hemosiderin is up to 35% iron. In the normal animal, non-viable RBCs are removed from the circulation by cells of the reticuloendothelial system in the liver, spleen and bone marrow. There, heme is broken down and the iron recycled for further use. In aged animals, when large amounts of iron are injected and rapidly cleared from the serum, or during chronic iron storage disease, the iron is preferentially deposited as hemosiderin thereby increasing intracellular concentrations and giving rise to the hemosiderosis that can be seen histologically (Underwood, 1977; Goyer and Clarkson, 2001).

## MECHANISM OF ACTION

For many functions, the body utilizes ferrous ( $\text{Fe}^{2+}$ ) iron to bind molecular  $\text{O}_2$ . In this way,  $\text{O}_2$  is transported by hemoglobin to cells in the peripheral tissues, myocytes bind  $\text{O}_2$  for intracellular utilization, mitochondrial proteins of the electron transport chain bind  $\text{O}_2$  for energy production and P450 enzymes bind  $\text{O}_2$  for its use in phase I metabolism of endogenous and xenobiotic chemicals. However, because of its reactivity, iron in its ferrous state must be carefully sequestered away to prevent the formation of highly ROS which can elicit severe cellular damage. Ferrous iron ( $\text{Fe}^{2+}$ ) and other transition metal ions,  $\text{Cu}^+$ ,  $\text{Cr}^{5+}$ ,  $\text{Ni}^{2+}$  or  $\text{Mn}^{2+}$ , can catalyze the formation of hydroxyl ion ( $\text{HO}^-$ ) and the extremely reactive and dangerous hydroxyl radical ( $\text{HO}^\bullet$ ) from the reduction of endogenous hydrogen peroxide ( $\text{HOOH}$ ) via the *Fenton reaction*:



The Fenton reaction causes site-specific accumulation of free radicals and initiates biomolecular damage. Free radicals are molecules or molecular fragments that contain one or more unpaired electrons in their outer orbital shell. If produced in great enough quantities to overwhelm the cellular antioxidant and radical-quenching protective mechanisms, hydroxyl radicals promote the formation of more hydroxyl radicals and other ROS such as superoxide. Superoxide combines with nitric oxide and forms peroxynitrite, which is as detrimental as hydroxyl radical. These ROS/RNS damage and destroy proteins and DNA by causing cross-linking, which inhibits their normal functions, or by initiating extensive damage and spontaneous degeneration of molecules such as lipids (Avery, 2011). ROS/RNS not only cause DNA damage, but also inhibit repair activities. ROS induce lipid peroxidation which if not quenched can initiate a chain reaction of lipid destruction and ROS formation

destroying vital cell membranes in mitochondria, nuclei and the cell periphery. Together, these effects can be of great enough magnitude to cause cell death, organ dysfunction and death. However, it is also of interest to note that iron is necessary for the normal functioning of macrophages and other leucocytes during the respiratory burst in inflammation to catalyze the formation of bactericidal hydroxyl radical (Gregus and Klaassen, 2001).

## TOXICITY

### General

Iron poisoning is not common in animals although potentially it could occur in any species. Clinical cases of acute iron toxicosis have been reported in dogs, pigs, horses, cattle and goats (Greentree and Hall, 1983; Ruhr *et al.*, 1983; Osweiler *et al.*, 1985; Holter *et al.*, 1990). Toxicity can occur through ingestion or parenteral administration. Because of their indiscriminate eating habits and close proximity to people and their nutritional supplements, dogs are the species most likely to ingest large quantities of iron-containing vitamins. Baby pigs are iron deficient at birth and require iron supplementation, which can result in peracute or acute toxicity. Limited cases of iron poisoning have occurred in horses and cattle through the use of iron supplements. In general, toxicity occurs in the GI mucosa (oral exposure), liver, myocardium and other tissues when the iron-binding capacity of the body is overwhelmed, and free iron causes oxidative damage. Genetic iron storage diseases are uncommon, but have been reported in mynah birds, toucans, Saler cattle, Egyptian fruit bats and rarely in horses.

### Acute toxicity in dogs, cattle, pigs and adult horses

Acute iron toxicity has been reported in dogs (Greentree and Hall, 1983), cattle (Ruhr *et al.*, 1983), pigs (Velasquez and Aranzazu, 2004), horses (Arnbjerg, 1981) and humans. In dogs, toxicosis primarily occurs through the accidental ingestion of large amounts of iron-containing vitamins or other iron supplements. In cattle, horses and pigs, it has occurred through accidental administration of excess amounts of iron supplements by oral or parenteral routes. Diagnosis is based on history, appropriate clinical signs and radiography in small animals as iron-containing pills are radiodense. In general, if the animals remain asymptomatic for greater than 8h following a single exposure, it is reported that they are unlikely to develop iron toxicity. In all species, ingestion of a toxic dose

(roughly greater than 20mg/kg in dogs) initially results in necrosis of the GI mucosal cells. This is followed by fluid loss, direct cardiotoxicity and widespread organ damage through the mechanisms described above. Fluid loss and decreased cardiac output can lead to circulatory shock. Iron toxicity has been described as occurring in four stages. Stage I occurs 0–6h post-ingestion and is characterized by vomiting, diarrhea, abdominal pain and depression. Stage II occurs from 6 to 24h post-ingestion and is characterized by apparent recovery. Stage III begins at 12–96h with commencement of additional vomiting, diarrhea, abdominal pain, GI hemorrhage, weakness, shock and possibly death. Stage IV, if it occurs, begins 2–6 weeks after ingestion and is characterized by GI fibrosis and obstruction (Greentree and Hall, 1983).

### Neonatal pigs

Paradoxically, iron toxicity in piglets resulting from oral supplementation or parenteral injection occurs because of the very low liver iron stores in newborn pigs and the need to supplement the small amounts of iron that they receive in sow's milk. Because of their low iron stores at birth, iron-deficiency anemia can occur in non-supplemented baby pigs within 2–4 weeks after birth. This is manifested clinically in the pigs as dyspnea, anorexia, increased infections and poor growth with some deaths. It is prevented by oral or parenteral injection of iron-containing compounds (Underwood, 1977; Osweiler *et al.*, 1985). When comparing routes of administration of the same iron compound the potential for toxicity is the greatest after intravenous injection followed by intramuscular injection with oral administration being the least toxic. In acute iron toxicosis of pigs, two syndromes are recognized. The first is a peracute syndrome which is characterized by sudden death minutes to a few hours after iron injection. In some ways this resembles an anaphylactic reaction in its rapidity of onset, vascular collapse and death, but the exact mechanism is not known. This peracute syndrome has also been reported in horses following administration of iron compounds (Lannek and Persson, 1972; Bergsjoe, 1974). The second syndrome described in pigs is a subacute to acute syndrome characterized by GI necrosis, severe depression, coma and death which can occur in the four stages described above. Pigs born to sows deficient in vitamin E and selenium are reported to be more susceptible to iron toxicosis (Osweiler *et al.*, 1985; Velasquez and Aranzazu, 2004).

### Neonatal horses

Although reports of iron toxicosis in horses and ponies are rare, several cases in the 1980s are of note. Newborn foals,



2–5 days old, were given an oral nutritional supplement containing ferrous fumarate. They became ill, icteric, weak and died of liver failure within 1–5 days following administration. The histologic lesions in the livers of these foals were remarkable in that they had the appearance of longer-term chronic lesions even though they were as little as 24 h old. These hepatic lesions consisted of prominent bile ductule proliferation, hepatic cell necrosis and periportal fibrosis (Divers *et al.*, 1983; Acland *et al.*, 1984). Through experimental administration, it was determined that ferrous fumarate, administered orally to foals within the first few days after birth, caused the liver failure seen in the earlier clinical cases (Mullaney and Brown, 1988). The increased sensitivity of the neonatal foals is thought to be from increased absorption in the GI tract and lower systemic iron-binding capacity (Poppenga, 2002). The iron overload in these foals resulting from oral administration of ferrous fumarate caused hepatic necrosis with bile ductule proliferation and fibrosis. It is speculated that what was histologically interpreted as proliferation of bile ductules was, in fact, proliferation of hepatic stem cells (oval cells) in response to the severe hepatic damage (personal observation). Experimentally, adult ponies have been administered iron as ferrous sulfate at 50 mg/kg/day orally for up to 8 weeks. Through the study and for 20 weeks after the end of dosing, no adverse clinical signs or hepatic damage were reported (Pearson and Andreasen, 2001).

### Iron storage disease

Iron storage disease resulting in hemosiderosis and hemochromatosis has been reported in several different species of animals. Hemochromatosis is the pathologic accumulation of iron in tissues, while hemosiderosis is the non-pathologic accumulation of iron. In birds, iron storage disease has been reported in mynah birds, toucans, birds of paradise and quetzals. The clinical signs are dyspnea, hepatic damage/insufficiency and death in mynah birds. Sudden death has been reported in toucans. It has been shown that the cause in mynah birds is due to maintenance of iron uptake from the GI tract, despite excess hepatic iron accumulation (Mete *et al.*, 2003). The treatment in birds can consist of a low iron diet with phlebotomy or the use of iron chelators (Rodenbusch *et al.*, 2004). A genetic, inheritable defect resulting in hemochromatosis, clinical wasting and skeletal defects can occur in Salers cattle beginning at 9–22 months of age (O'Toole *et al.*, 2001; Norrdin *et al.*, 2004). Egyptian fruit bats (*Rousettus aegyptiacus*) are also known to have genetic iron storage disease. In these bats, it was found that if the sum of transferrin saturation and serum iron was greater than 51, the individual bat had

a high probability of having iron overload. When the sum was greater than 90, there was a high probability of having hemochromatosis (Farina *et al.*, 2005). In addition, although rarely reported, hemochromatosis with liver damage has also been reported in horses (Pearson *et al.*, 1994) and captive northern fur seals (Mazzaro *et al.*, 2004). Finally, one case of hemochromatosis secondary to repeated blood transfusions every 6–8 weeks for 3 years for the treatment of red cell aplasia has been reported in an aged miniature schnauzer (Sprague *et al.*, 2003).

## TREATMENT

Treatment of iron toxicity varies with the inciting cause, dose and duration of the disease. General therapy is to limit absorption (although activated charcoal is ineffective at binding iron), provide symptomatic and supportive care, remove gastric bezoars of sticky iron-containing pills, surgically if necessary, and increase excretion. Because the body has limited ability to excrete excess iron (other than through bleeding), urinary excretion can be enhanced through the use of a chelating agent. A specific chelator of iron, deferoxamine has been used in the treatment of iron toxicity. Deferoxamine has a strong affinity for iron, a low affinity for calcium and competes effectively for iron in ferritin and hemosiderin, but not in transferrin, hemoglobin or heme-containing enzymes. It is poorly absorbed orally and so is given parenterally by slow intravenous drip. Since it is excreted primarily by the kidneys, it should be given carefully to patients with renal insufficiency (Osweiler *et al.*, 1985; Goyer and Clarkson, 2001; Poppenga, 2002).

## CONCLUDING REMARKS

Iron is essential for normal physiological functioning. Iron-deficiency anemia is common in humans and also occurs in piglets, veal calves and parasitized animals. Therefore, iron supplements are readily available and widely used. In addition, some species have a propensity for genetic iron storage diseases. Fortunately, both acute and chronic iron toxicoses are rare in animals. When toxicosis does occur, cellular damage is caused by the presence of free iron in excess of the body's capacity to bind and sequester it. The free iron then initiates the generation of ROS in excess of cellular oxidant defenses resulting in lipid peroxidation of membranes, protein and DNA cross-linking, and cell death.

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# Lead

Larry J. Thompson

## INTRODUCTION

Lead is a bluish white to gray heavy metal that was probably the first toxic element recognized by man and yet still has great relevance today. The chemical symbol for lead, Pb, is short for the Latin word *plumbum*, meaning liquid silver. The main source of lead is the ore named galena, which contains lead sulfide. The main use of lead today is in lead-acid storage batteries but historically it has had widespread usage in paints (white, yellow and red pigments) and as a gasoline additive (tetraethyl lead) although these latter uses have essentially been phased out. A debate still continues as to the role lead played in the fall of the Roman Empire. Although lead was indeed used in some Roman water pipes (the word *plumbum* leading to our modern word plumber) the main source of lead for the Romans was probably sapa, a syrup used to sweeten wine and preserve fruit, which was made by boiling grape juice in lead pots thereby adding lead acetate to the liquid. The use of lead continues in our modern world with lead-containing solder in our electronics as well as lead-containing glass, from cathode ray tube monitors to fine crystal. Among all the metals, lead poisoning is encountered with greatest frequency in certain species of animals and poses a serious concern to animal health. This chapter describes the toxicity of lead in mammalian and avian species.

## BACKGROUND

Lead is a toxic element and has not been shown to be an essential trace element for nutrition. The historical use of lead in gasoline, paint, construction materials and many

other products has resulted in lead being one of the most significant environmental contaminants in the world. Additional sources of lead have included lead weights (e.g., for fishing or curtains), small lead trinkets and toys, lead shot and bullets for weapons, lead arsenate pesticides and many other products as well as single source environmental contamination from mining, smelting and recycling operations. As a result of increased regulation of lead and the decreasing use of lead-containing products, the overall incidence of lead poisoning (also called plumbism) in animals and humans has been decreasing. While environmental contamination with lead does not resolve readily, the overt poisoning of domestic animals from environmental sources (e.g., from contaminated forages, plants, water or other food sources) has been decreasing in number. Still, lead poisoning in animals is encountered with greatest frequency compared to any other metal.

## TOXICOKINETICS

The main route of entry of lead into the body is the digestive tract with absorption dependent upon the chemical form of lead and the physiological state of the animal. Organic lead compounds are, in general, more readily absorbed than either inorganic lead salts or the metallic form of lead. Dermal absorption of organolead compounds can be significant but the salt or metallic forms of lead are not absorbed dermally. The fumes from heated lead or very fine particles ( $<0.5\mu\text{m}$ ) of lead can enter the lung alveoli and be absorbed with the larger particles lodging in the ciliated portion of the bronchial tree. These larger particles can be transported up by mucociliary action and then swallowed with absorption through

the gastrointestinal tract. Fine particles of lead and lead salts can be solubilized in the acid environment of the stomach and the small intestine is the site of most lead absorption. Absorption of lead from the alimentary tract is usually influenced by dietary factors and by the size of lead particles. High dietary fat and mineral deficiency can increase lead absorption by seven- and 20-fold, respectively. In an experimental study, dogs maintained on a high-fat, low-calcium diet absorbed significantly more lead than those kept on a balanced diet (Hamir *et al.*, 1988). Young animals absorb a larger portion of the lead from the gastrointestinal tract than do adults. Animals with a calcium deficiency have increased absorption of lead. Pregnancy or lactation as well as deficiencies of iron, zinc or vitamin D can also enhance lead absorption. Lead crosses the placental barrier and the residue can be detected in significant amounts in fetal blood and organs. Among the fetal organs, the highest concentrations are found in the blood and liver (Kelman and Walter, 1980; O'Hara *et al.*, 1995; Flora *et al.*, 2011). Lead also passes through the milk.

Nonruminant animals absorb approximately 10% of dietary lead, and ruminants absorb less than 3%. Young animals can absorb up to 90% of the ingested lead. Following absorption, a large proportion of lead is carried on erythrocyte membranes (60–90%, species dependent) with most of the remainder of lead bound to protein or sulfhydryl compounds, with only a very small proportion found free in the serum. Lead is widely distributed in the body, including crossing the blood–brain barrier (Seimiya *et al.*, 1991). In the soft tissues lead binds to various proteins as well as metallothionein but accumulates in the active bone matrix (about 90%) serving as a relatively inert reservoir of lead in the body. This reservoir can be mobilized by lactation, pregnancy or the action of chelating agents. Otherwise lead has a very slow turnover rate from the bone. Lead is normally very slowly excreted via the bile with very little in the urine. Chelation therapy greatly increases the urinary output of lead.

## MECHANISM OF ACTION

Lead interferes with several biochemical processes in the body by binding to sulfhydryl and other nucleophilic functional groups causing inhibition of several enzymes and changes in calcium/vitamin D metabolism. Lead also contributes to oxidative stress within the body. Lead inhibits the body's ability to make hemoglobin by interfering with several enzymatic steps in the heme pathway. Specifically, lead decreases heme biosynthesis by inhibiting delta-aminolevulinic acid dehydratase and ferrochelatase activity. These changes contribute to the anemia

that develops in chronic lead poisoning. An increased fragility of red blood cells also contributes to the anemia.

From various experimental studies, biochemical and pathological evidence suggests that lead is neurotoxicant, as it significantly disrupts certain brain structures and functions. High-dose exposure to lead (i.e., blood levels in excess of 4  $\mu$ M) disrupts the blood–brain barrier. Molecules such as albumin that normally are excluded freely enter the brain of immature animals exposed to these concentrations of lead (Clasen *et al.*, 1973; Goldstein *et al.*, 1974; Bressler and Goldstein, 1991). Ions and water follow and edema is produced. Intracranial pressure rises as edema accumulates in the brain because of the physical restraint of the skull. When the intracranial pressure approaches the systemic pressure, cerebral perfusion decreases and brain ischemia occurs.

Many of the neurotoxic effects of lead appear related to the ability of lead to mimic or in some cases inhibit the action of calcium as a regulator of cell function (Bressler and Goldstein, 1991). At a neuronal level, exposure to lead alters the release of neurotransmitters (dopamine, acetylcholine and  $\gamma$ -aminobutyric acid) from nerve endings. Spontaneous release is enhanced and evoked release is inhibited. The former may be due to activation of protein kinases in the nerve endings and the latter to blockade of voltage-dependent calcium channels.

Brain homeostatic mechanisms are disrupted by exposure to higher levels of lead. The final pathway appears to be a breakdown in the blood–brain barrier. Again, the ability of lead to mimic or mobilize calcium and activate protein kinases may alter the properties of endothelial cells, especially in immature brain, and disrupt the barrier. In addition to a direct toxic effect upon the endothelial cells, lead may alter indirectly the microvasculature by damaging the astrocytes that provide signals for the maintenance of blood–brain barrier integrity, and necrosis in neurons with shrunken cytoplasm, pyknotic nuclei and increased perineuronal space.

Recent studies provided evidence of increased production of reactive oxygen species (ROS) following lead exposure. Lead induced oxidative damage in several tissues by enhancing lipid peroxidation through Fenton reaction or by direct participation in free radical-mediated reactions, such as inhibition of  $\delta$ -aminolevulinic acid dehydratase (ALAD) activity or accumulation of ALA, a metabolite that can release  $\text{Fe}^{2+}$  from ferritin, and induce oxidative damage.

## TOXICITY

Mammals, birds and reptiles have all been found to develop lead poisoning. The toxic dose of lead has been



determined for several species but is difficult to apply to clinical cases where the exposure history is unclear (Gwaltney-Brant, 2004). In general, young animals are more susceptible to lead toxicosis because they are more prone to lead pica and have a higher rate of absorption (about 90%) from the intestinal tract. Cattle have been most widely reported with lead toxicosis, probably due to their propensity to ingest discarded lead-acid batteries and construction materials including paints. Dogs are also commonly reported with lead toxicosis, probably due to their chewing habits and ingestion of small lead objects around the house. Both cats and dogs have been exposed to lead by the renovations of older homes containing leaded paints. The main route seems to be the ingestion of fine dust by the grooming habits of indoor pets and their tendency to ingest small objects.

Clinical signs of lead toxicosis vary with the species involved, duration of exposure and amount of lead absorbed. The major systems affected by lead poisoning are the gastrointestinal system, central nervous system and hematological system. Abdominal pain and diarrhea can be common clinical signs in animals exposed to excess lead. Anorexia is common as well as vomiting in those species that are able to. Neurological signs including depression, weakness and ataxia can progress to more severe clinical signs of muscle tremors or fasciculations, head pressing (especially in ruminants), blindness, seizure-like activity and death. Many animals with chronic lead poisoning will show subtle and non-specific clinical signs such as abdominal discomfort, vague gastrointestinal upsets, anorexia, lethargy, weight loss and behavior changes. Horses develop acute lead toxicosis and show clinical signs of laryngeal paralysis and "roaring," in addition to colic and seizure-like activity. Evidence suggests that horses may be more susceptible to chronic lead toxicosis than cattle. Horses exposed to daily intake as low as 1.7 mg/kg body weight (approximately 80 ppm Pb in forage dry matter) were poisoned (Aronson, 1972). Clinical signs of lead toxicosis in avians vary with waterfowl and raptors mainly displaying a chronic wasting disorder with apparent peripheral neuropathy. Psittacines are more likely to display gastrointestinal problems and neurological abnormalities.

Gross lesions in animals dying of lead poisoning are often minimal and non-specific, although lead-containing objects may be visible in the gastrointestinal tract. Histologically, there may be degeneration and necrosis of the renal tubular epithelium or the presence of acid-fast inclusion bodies (Hamir *et al.*, 1988; O'Hara *et al.*, 1995). Brain lesions in a calf poisoned with lead included multiple focal or laminar lesions of neuronal necrosis in the cerebral cortex, caudatum and medial nuclei of thalamus, predominantly at the tips of gyri in the occipital and parietal lobes. The lesions spread occasionally to the deeper region of the gyri along the

sulci (Seimiya *et al.*, 1991). The affected neurons were shrunken and angular, sometimes triangular in outline with pale eosinophilic cytoplasm. The nuclei showed pyknosis and rhexis. Edematous dilation of perivascular and perineuronal spaces with spongiotic state of neuropil was observed from the molecular layer to outer zone of the white matter. Astrocytic proliferation was also observed. Blood capillaries were congested with enlarged and increased endothelial cells. Meningeal blood vessels were prominently congested with mild lymphocytic infiltration. Edema of Purkinje cell layer in the cerebellum and mild neuronal degeneration in the nucleus of mesencephalon were seen.

Lead is also a reproductive and developmental toxicant and details can be found in a recent publication (Flora *et al.*, 2011).

Diagnosis of lead poisoning in animals should be made with a combination of history, clinical or necropsy findings, and lead analysis of tissue. Basophilic stippling of erythrocytes and inhibition of hemoglobin synthesis are characteristic hematological features of lead poisoning. From a living animal, whole blood is the best sample for laboratory determination of lead. The normal background concentration of lead in the blood of mammals is below 0.1 ppm. With clinically affected animals, lead concentrations above 0.35 ppm are compatible with a diagnosis of lead toxicosis. Postmortem samples of choice are kidney and liver with lead concentrations above 10 ppm on a wet weight basis being diagnostic for lead toxicosis in domestic species.

## TREATMENT

Acute lead poisoning in animals is usually fatal if the animals are not treated promptly. Treatment approach for lead poisoning in animals includes stabilizing and supporting the animal especially if severe clinical signs are present, preventing additional exposure to lead and chelation therapy to quickly reduce the body burden of lead. The exposure history of the animal should be reviewed for potential sources of lead and the need for gastrointestinal decontamination. The use of chelating agents when large amounts of lead are present in the gastrointestinal tract may actually enhance the absorption of lead into the body. Physical removal of lead-containing objects by surgical means may be necessary with larger objects. The parenteral use of calcium disodium ethylenediaminetetraacetic acid (CaEDTA) has been commonly used for several decades as a chelation agent in domestic animals (Kowalczyk, 1984).

Although other chelators may be superior, CaEDTA is still widely used in veterinary medicine, especially

in large animals. CaEDTA is given intravenously (IV) or subcutaneously (SQ) and chelates and mobilizes the lead from bone resulting in a transient increase in blood lead levels. This increase in blood lead can increase soft tissue lead levels leading to an exacerbation of clinical signs. Preceding CaEDTA usage with a chelator that specifically targets lead in the soft tissue (e.g., British Anti-Lewisite or BAL) has been recommended but is difficult to accomplish in most practice settings. CaEDTA can be nephrotoxic, especially in situations where the animal is dehydrated. Recommended treatment with CaEDTA for large animals is 73 mg/kg/day, divided into two or three doses given over the course of a day given by slow IV. For example, a 6.6% solution of CaEDTA (in normal saline or 5% dextrose) can be given IV at a rate of 1 ml per 2 pounds (0.9 kg) of body weight per day, in divided doses. Treatment should continue for 3–5 days. If additional treatment is needed, a rest period of 2 days with continued supported care is suggested before the additional 3–5-day second treatment period. An alternative treatment regimen is to administer CaEDTA at 110 mg/kg IV twice daily for 2 days. If additional treatment is needed, first apply the 2-day rest period of supportive care before initiating the second treatment period of 2 days at 110 mg/kg twice daily. Thiamine has been shown to be a valuable adjunct to the treatment of lead poisoning in ruminants (Bratton *et al.*, 1981) and is recommended for other species as well. A dose of 2 mg/kg/day for calves and 250–2000 mg/day for adult cattle has been recommended.

If commercial CaEDTA is unavailable, a stock solution can be formulated for emergency antidotal usage. A 10% stock solution can be made by dissolving 101.1 g of tetrasodium EDTA (Na<sub>4</sub>EDTA) plus 30 g of anhydrous calcium chloride (CaCl<sub>2</sub>) in distilled water to a final volume of 1000 ml. From the stock solution a working 2.22% solution can be made by mixing 220 ml of the 10% stock solution with 780 ml distilled water. Using the 2.22% solution, the daily dosage of 73 mg/kg/day is equal to approximately 3.5 ml/kg of body weight. This should be divided into two or three separate administrations (Thompson, 1992). Tetrasodium EDTA should never be administered by itself as it may cause hypocalcemia.

Recommended treatment with CaEDTA for dogs is 100 mg/kg/day in four divided doses. Treatment should continue for 2–5 days and a second round of treatment is rarely needed but a 5-day rest period is recommended before applying additional treatment. CaEDTA concentration should be 10 mg/ml and may be administered by slow IV or by SQ route. Cats can be treated with 27.5 mg in 15 ml normal saline or 5% dextrose SQ every 6 h for 5 days or the same dose as a slow IV infusion.

Succimer (meso-2,3-dimercaptosuccinic or DMSA) is an orally administered chelating agent that is less likely to have adverse side effects associated with CaEDTA. The recommended treatment in dogs is an oral dose of

succimer at 10 mg/kg, repeated three times daily for 10 days (Ramsey *et al.*, 1996). Succimer has also been used orally in caged birds at a dose of 25–35 mg/kg twice daily for 5 days. Several weeks of therapy may be needed in avians. In initial experimental work, succimer given to experimentally lead-poisoned calves IV at 25 mg/kg/day for 4 days was more effective than CaEDTA at decreasing lead concentrations in the liver and kidney (Meldrum and Ko, 2003).

In a recent experimental study, Pachauri *et al.* (2009) provided evidence of the efficacy of combinational therapy using an antioxidant with a thiol chelator in reversing neurological dystrophy caused by chronic lead exposure in rats.

## CONCLUDING REMARKS

While cases of lead toxicosis in animals have been decreasing, it should remain on the clinician's list of rule-outs for seizure-like activity, blindness and vague neurological and gastrointestinal disorders. At a minimum, the environment of the animal should be reviewed for possible lead sources.

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# Manganese

*Dejan Milatovic and Ramesh C. Gupta*

## INTRODUCTION AND BACKGROUND

Manganese (Mn) was discovered by Sheele in Sweden in 1774. Mn has an atomic number of 25 and has two oxidation states: Mn(0) and Mn<sup>2+</sup>. It does not occur naturally in a pure state or as a metal, but as a component of over 100 minerals (ATSDR, 2000). The most important Mn-containing minerals are oxides, carbonates and silicates (Post, 1999). Mn is abundantly found in the environment, including rocks, soil, water and food, with the highest concentrations typically in nuts, cereals, grains, fruits, vegetables and tea. Mn is a component of certain aluminum alloys, particularly those used in the manufacture of soft drink cans. Inorganic Mn is also used in dry cell batteries, animal feed, brick coloring and fertilizers (ATSDR, 2000; Corathers, 2001). Organic Mn is used as a fuel additive and in fungicides, such as macozebe and maneb (ATSDR, 2000; Kaiser, 2003).

Mn is an essential element for maintaining the proper function and regulation of many biochemical and cellular reactions (Takeda, 2003) that are critical for humans, animals and plants. It is required for growth and development and plays a role in immune response, blood sugar homeostasis, adenosine triphosphate (ATP) regulation, digestion, bone growth, reproduction and lactation. It is a necessary component of numerous metalloenzymes, such as Mn superoxide dismutase, arginase, phosphoenol-pyruvate carboxylase and glutamine synthase (Aschner and Aschner, 2005). As a result, dietary deficiency of Mn is known to affect several enzymes, including glycosyltransferases, xylosyltransferases, arginase and mitochondrial superoxide dismutase (NRC, 2005). The dietary requirement of Mn is

10ppm in rats and mice, 40ppm in guinea pigs, 20ppm in pigs, 28–30ppm in chickens, 20ppm in beef cattle and 40ppm in dairy cattle. Practically, Mn deficiency occurs more frequently in cattle, pigs and poultry. In ruminants, Mn deficiency can be linked to silent heat, reduced conception, abortions, reduced birth weight, an increased percentage of male calves, paralysis and skeletal damage in calves. Mn deficiency can cause delayed estrus, reduced fertility and spontaneous abortions in mares. Foals are born with skeletal deformities and muscle contractures, such as asymmetry of the skull, curvature of the vertebral column, shortened limb bones, enlarged joints and contracture of neck muscles. In dogs, Mn deficiency can cause crooked and shortened soft bones (Puls, 1994).

Despite its essentiality, Mn overexposure can cause a variety of toxic effects in humans and animals. Mn has been linked to a peculiar extrapyramidal syndrome in occupational workers since 1837. Since then, incidences of Mn poisoning, especially in mines and steel factories, have been reported in many countries. Fortunately, in the last three decades there has been a declining trend even in developing countries. Mn poisoning can occur in animals or humans if the exposure level of Mn exceeds the normal levels in the environment. Toxicity can also occur if either the dietary concentration exceeds the maximal tolerable levels or if dietary intake of other minerals is marginal (Hartman *et al.*, 1955; Ivan and Hidiroglou, 1980; Southern and Baker, 1983; Baker and Halpin, 1991; Hauser *et al.*, 1994). The brain, in particular, is highly susceptible to Mn toxicity. Excessive accumulation of Mn in specific brain areas, such as the substantia nigra, the globus pallidus and the striatum, produce neurotoxicity leading to a degenerative brain disorder, referred to as



manganism. In addition to neurological effects, Mn accumulation is also associated with reproductive and developmental effects. High-dose exposure to Mn has been associated with increased fetal brain Mn concentrations (Kontur and Fechter, 1985) although several studies have reported an ability of the placenta to reduce systemic delivery of Mn to the fetal brain. Moreover, Mn plays a role in the modulation of the immune system, and in protein, lipid and carbohydrate metabolism (Address *et al.*, 1997; Malecki *et al.*, 1999; Aschner *et al.*, 2002; Fitsanakis and Aschner, 2005). This chapter describes deficiency and toxicity of Mn in animals.

## PHARMACOKINETICS/ TOXICOKINETICS

Absorption of Mn is via oral and inhalation routes. Absorption of Mn through the skin is negligible. Apparent absorption of orally administered  $^{54}\text{Mn}$  has been estimated to range from 1 to 5% in rats, humans and livestock (Hurley and Keen, 1987; Davis *et al.*, 1993). In young rats, Mn absorption has been reported to be 8%. Absorption of Mn appears to occur by a low-capacity saturable process and by diffusion (Garcia-Aranda *et al.*, 1983; ATSDR, 2000). Uptake and retention of dietary Mn was found to be greater in suckling than post-weaning rats (Keen *et al.*, 1986). In cattle, using  $\text{MnSO}_4$  as 100% available,  $\text{MnO}$  is 58%,  $\text{MnO}_2$  is 33% and  $\text{MnCO}_2$  is 28% available (Puls, 1994). Mn is more available from hay to cattle than from silage. It is important to note that newborn calves absorb and retain more dietary Mn than adults.

Absorption of Mn can be influenced by various dietary factors, such as iron, calcium, phosphorus, phytate and amino acids. Henry (1995) estimated the relative bioavailability of Mn to poultry was 0.55 from manganese carbonate, 0.3 from manganese dioxide and 0.75 from manganese monoxide, when the bioavailability of manganese from manganese sulfate and manganese chloride was considered to be 1. Manganese from manganese carbonate, dioxide and monoxide (relative bioavailabilities were 0.3, 0.35 and 0.6, respectively) was even less available to sheep than manganese sulfate (relative bioavailability was 1).

Following absorption, Mn can be distributed to many organs. The average adult human has about 12 mg of Mn, of which about 43% resides in the skeletal system and the rest in soft tissues including the liver, pancreas, kidneys and central nervous system (CNS). Such information about Mn is not available for animals. It is interesting to note that in dogs, the pancreas accumulates 3–60 times more Mn than other soft tissues. Furthermore, colored hair has higher Mn levels than non-colored hair.

After absorption from the gut, Mn is transported to the liver by  $\alpha_2$ -macroglobulins and albumin (Andersen *et al.*, 1999). This protein-bound Mn is efficiently cleared in the liver and is bound to transferrin. Transferrin is believed to be the primary transporter of Mn across the blood–brain barrier (BBB) and Mn is taken up into astrocytes and neurons in the brain. Astrocytes serve as the major homeostatic regulator and storage site for Mn in the brain. Increased accumulation of Mn in astrocytes may alter release of glutamate and elicit excitatory neurotoxicity (Erikson and Aschner, 2003). Neuronal uptake of Mn involves transferrin (Suarez and Eriksson, 1993) and utilization of specific transporter systems, such as the dopamine transporter (Chen *et al.*, 2006a; Anderson *et al.*, 2007). At the subcellular level, Mn preferentially accumulates in mitochondria, where it disrupts oxidative phosphorylation and increases the generation of reactive oxygen species (ROS) (Gunter *et al.*, 2006).

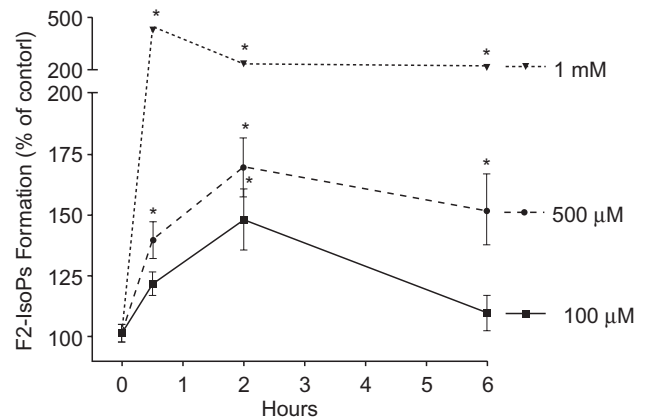
Rodent studies of enhanced CNS uptake during development have been contradictory. While one study indicates that the amount of Mn that crosses the placenta was not increased by enhanced maternal exposure via diet (Jarvinen and Ahlstrom, 1975), another study indicated increased neonatal brain Mn following chronic high-level exposure of the dam to Mn in drinking water throughout gestation (Kontur and Fechter, 1985). Other studies also showed that, when compared with adults, neonatal rodents attain higher brain Mn levels following similar oral exposures (Kontur and Fechter, 1985; Dorman *et al.*, 2000). This tendency of neonates to attain higher brain Mn concentrations may reflect a less than optimal BBB, markedly reduced biliary Mn excretion rates, and/or increased placental Mn concentration (Aschner and Aschner, 2005). However, an increase in the placental concentrations does not necessarily mean higher placental transfer of Mn to the fetus or higher fetal exposure (Dorman *et al.*, 2005; Yoon *et al.*, 2009a). Furthermore, brain Mn concentrations are higher in developing animals, suggesting that high amounts of Mn are required for normal brain development in infants (Keen *et al.*, 1986; Takeda *et al.*, 1999). Therefore, whether the relatively higher net increase in brain Mn observed in neonates compared to adults would pose an increased risk for neurotoxicity requires further understanding of the Mn requirements for normal brain development (Yoon *et al.*, 2009b).

The absorbed Mn can be excreted via urine and bile. Excretion of Mn through urine is minor. Bile is the major excretory route of injected or ingested Mn. Rats excreted 15–40% (Ballatori *et al.*, 1987) and calves excreted 21% (Abrams *et al.*, 1977) of injected doses of Mn in bile. In humans, Mn is eliminated almost entirely with feces, and only 0.1–1.3% of daily intake through urine. Klaassen (1974) observed that rats excreted proportionately more Mn into bile than rabbits and dogs. Following ingestion, effectiveness of the gut in preventing excess

absorption blunts the effect of biliary excretion (Abrams *et al.*, 1977; Davis *et al.*, 1993; Malecki *et al.*, 1996). Calves excreted 0.2% of a duodenal dose of Mn into bile in one study (Abrams *et al.*, 1977) and 2.1–3.6% of high levels of Mn infused intraduodenally in another study (Symonds and Hall, 1983).

## MECHANISM OF ACTION

Mn is generally described as a neurotoxicant, selectively affecting basal ganglia structures. Although it is known that Mn is a cellular toxicant which can impair the transport system, enzyme activity and receptors function, the principal mechanism by which Mn neurotoxicity occurs has not yet been clearly established (Aschner and Aschner, 1991; Aschner *et al.*, 2007). Since mitochondria are the principal intracellular repository for metals (Cotzias and Greenough, 1958), binding of Mn to inner mitochondrial membrane or matrix proteins (Gavin *et al.*, 1990) directly interacts with proteins involved in oxidative phosphorylation. Mn directly inhibits complex II (Singh *et al.*, 1974) and complexes I–IV (Zhang *et al.*, 2003) in brain mitochondria, and suppresses ATP-dependent calcium waves in astrocytes, suggesting that Mn promotes potentially disruptive mitochondrial sequestration of calcium (Tjalkens *et al.*, 2006). Elevated matrix calcium increases the formation of ROS by the electron transport chain (ETC) (Kowaltowski *et al.*, 1995) and results in inhibition of aerobic respiration (Kruman and Mattson, 1999). Recent studies with primary astrocytes and neurons have shown that Mn exposure induces an increase in the biomarkers of oxidative stress (Milatovic *et al.*, 2007, 2009). Measurement of F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs) (Morrow and Roberts, 1999; Milatovic and Aschner, 2009), a group of arachidonic acid-derived prostanoid isomers generated by free radical damage due to arachidonic acid, revealed that astrocytes exposed to Mn at neurotoxic level (100  $\mu$ M, 500  $\mu$ M or 1 mM) induced significant elevations in F<sub>2</sub>-IsoPs (Figure 38.1). Thus, increases in ROS, which are generated by electron leak from the ETC (Turrens and Boveris, 1980), potentially damaging mitochondria directly or through the effects of secondary oxidants like superoxide, H<sub>2</sub>O<sub>2</sub> or peroxynitrite (ONOO<sup>-</sup>), mediate Mn-induced oxidative damage. Moreover, superoxide produced in the mitochondrial electron transport chain may catalyze the transition shift of Mn<sup>2+</sup> to Mn<sup>3+</sup> through a set of reactions similar to those mediated by superoxide dismutase and thus lead to the increased oxidant capacity of this metal (Gunter *et al.*, 2006). Consequent oxidative damage produces an array of deleterious effects: it may cause structural and functional derangement of the phospholipids bilayer of membranes, disrupt energy metabolism, metabolite biosynthesis,



**FIGURE 38.1** Effects of MnCl<sub>2</sub> on F<sub>2</sub>-IsoPs formation in cultured astrocytes. Rat primary astrocyte cultures were incubated at 37°C in the absence or presence of MnCl<sub>2</sub> (100  $\mu$ M, 500  $\mu$ M or 1 mM), and F<sub>2</sub>-IsoPs levels were quantified at 30 min, 2 h and 6 h. Data represent the mean  $\pm$  S.E.M. from three independent experiments. \*Significant difference between values from control and Mn-treated astrocytes (\**p* < 0.05).

calcium and iron homeostasis and initiate apoptosis (Attardi and Schatz, 1988; Yang *et al.*, 1997; Uchida, 2003).

Consistent and preceding the Mn-induced increased in biomarkers of oxidative damage (F<sub>2</sub>-IsoPs) (Figure 38.1), Milatovic *et al.* (2007) demonstrated an early decrease in astrocytic ATP levels. As a consequence, ATP depletion or a perturbation in energy metabolism might diminish the ATP-requiring neuroprotective action of astrocytes, such as glutamate and glutamine uptake and free radical scavenging (Rao *et al.*, 2001). In addition, depletion of high-energy phosphates may affect intracellular Ca<sup>2+</sup> in astrocytes through mechanisms involving the disruption of mitochondrial Ca<sup>2+</sup> signaling. This assertion is supported by data showing that Mn inhibits Na<sup>+</sup>-dependent Ca<sup>2+</sup> efflux (Gavin *et al.*, 1990) and respiration in brain mitochondria (Zhang *et al.*, 2004), both critical for maintaining normal ATP levels and ensuring adequate inter-mitochondrial signaling. Decrease in ATP following Mn exposure is also associated with excitotoxicity, suggesting a direct effect on astrocytes with subsequent impairment of neuronal function. Mn down-regulates the L-glutamate/L-aspartate transporter in astrocytes (Erikson and Aschner, 2002) and decreases levels of glutamine synthase in exposed primates (Erikson *et al.*, 2008). Studies with a neonatal rat model indicated that both pinacidil, a K<sup>+</sup> channel agonist, and nimodipine, a Ca<sup>2+</sup> channel antagonist, reversed Mn neurotoxicity and loss of glutamine synthase activity, further indicating excitotoxicity in the mechanism of Mn-induced neurotoxicity. Excessive Mn may lead to excitotoxic neuronal injury both by decreased astrocytic glutamate uptake and by loss of ATP-mediated inhibition of glutamatergic synapses.

Another consequence of Mn-associated increased oxidative stress and mitochondrial energy failure is the induction of the mitochondrial permeability transition (MPT), a  $\text{Ca}^{2+}$ -dependent process characterized by the opening of the permeability transition pore (PTP) in the inner mitochondrial membrane. This process results in increased permeability to protons, ions and other solutes (Zoratti and Szabo, 1995), which subsequently leads to a collapse of the mitochondrial inner membrane potential ( $\Delta\Psi_m$ ). Loss of the  $\Delta\Psi_m$  results in colloid osmotic swelling of the mitochondria matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis and further generation of ROS. Researchers have shown a concentration-dependent effect of Mn on the mitochondrial inner membrane potential in cultured astrocytes (Rao and Norenberg, 2004; Milatovic *et al.*, 2007). Zhang *et al.* (2004) revealed that high levels of Mn chloride (1 mM) cause a significant dissipation of the  $\Delta\Psi_m$  in isolated rat brain mitochondria, consistent with induction of the MPT.

Oxidative stress as an important mechanism in Mn-induced neurotoxicity has also been confirmed in the *in vivo* model. Analyses of cerebral biomarkers of oxidative damage revealed that a one-time challenge of mice with Mn (100 mg/kg) was sufficient to produce significant increases in  $\text{F}_2$ -IsoPs (Table 38.1) 24 hours following the last injection. Increased striatal concentrations of ascorbic acid and glutathione (GSH), antioxidants that when increased signal the presence of an elevated burden from ROS, as well as other markers of oxidative stress, have been previously reported (Desole *et al.*, 1994; Dobson *et al.*, 2004; Erikson *et al.*, 2007). Mn-induced decrease in GSH and increased metallothionein was reported in rats (Dobson *et al.*, 2003) and nonhuman primate studies (Erikson *et al.*, 2007). ROS may act in concert with reactive nitrogen species (RNS) derived from astroglia and microglia to facilitate the Mn-induced degeneration of dopaminergic (DAergic) neurons. DAergic neurons possess reduced antioxidant capacity, as evidenced by low intracellular GSH, which renders these neurons more vulnerable to oxidative stress and glial activation relative to other cell types (Sloot *et al.*, 1994; Greenamyre *et al.*, 1999). Therefore, the overactivation of glia and release of additional neurotoxic factors may represent a crucial component associated with the degenerative process of DAergic neurons.

Mn-induced ROS generation is also associated with inflammatory responses and release of inflammatory mediators, including prostaglandins. Recent studies confirmed that in parallel with an increase in biomarkers of oxidative damage, Mn exposure also induced an increase in biomarkers of inflammation, prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ), *in vitro* and *in vivo* (Milatovic *et al.*, 2007, 2009). Results from an *in vivo* study showed that Mn exposure induced a time-dependent increase in  $\text{PGE}_2$

TABLE 38.1 Cerebral  $\text{F}_2$ -IsoPs and  $\text{PGE}_2$  levels in saline (control) or MnCl<sub>2</sub> (100 mg/kg, sc) exposed mice. Brains from mice exposed once or three times (day 1, 4 and 7) to MnCl<sub>2</sub> were collected 24 h post last injection

Exposure	$\text{F}_2$ -IsoPs (ng/ g tissue)	$\text{PGE}_2$ (ng/g tissue)
Control (saline)	3.013 + 0.03939	9.488 + 0.3091
Single Mn	4.302 + 0.3900*	12.03 + 0.4987*
Multiple Mn	4.211 + 0.4013*	14.22 + 1.019*

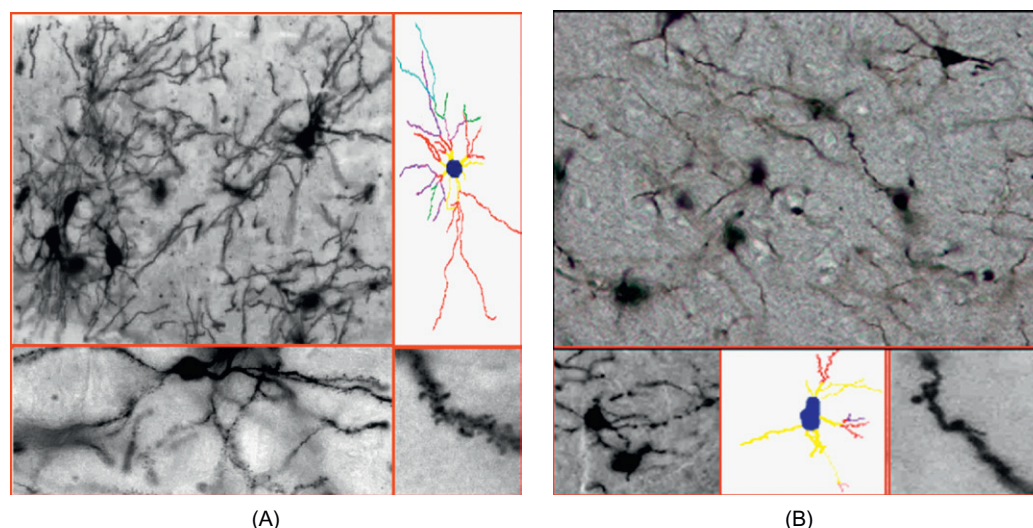
Values of  $\text{F}_2$ -IsoPs represent mean  $\pm$  SEM ( $n = 4-6$ ).

\*Significant difference between values from control and Mn-treated mice (\* $p < 0.05$ ).

(Table 38.1). Recent studies have also shown an inflammatory response of glial cells following Mn exposure (Chen *et al.*, 2006b; Zhang *et al.*, 2009; Zhao *et al.*, 2009). Mn potentiates lipopolysaccharide-induced increases in proinflammatory cytokines in glial cultures (Filipov *et al.*, 2005) and increases in nitric oxide production (Chang and Liu, 1999). An increase in proinflammatory genes, such as tumor necrosis factor- $\alpha$ , iNOS and activated inflammatory proteins such as P-p38, P-ERK and P-JNK have been measured in primary rat glial cells after Mn exposure (Chen *et al.*, 2006b). However, data from a recent study indicate that release of proinflammatory mediators following Mn exposure is not only associated with glial response, but neurons as well, and suggests that these two events are mechanistically related, with neuroinflammation either alone or in combination with activated glial response contributing to oxidative damage and consequent cell injury.

Dysregulation of excitatory glutamatergic neurotransmission by Mn is also associated with DAergic and GABAergic neuronal dysfunction. It is known that *in vitro* Mn can promote autooxidation of dopamine, which leads to the creation of reactive quinones (Miller *et al.*, 1990; Shen and Dryhurst, 1998). However, rodent and nonhuman primate data offer conflicting evidence on the influence of Mn exposure on catecholamine concentrations (Olanow *et al.*, 1996; Struve *et al.*, 2007). Additional evidence from nonhuman primate data suggests an Mn-induced postsynaptic decrease of D2-like dopamine receptor levels (Eriksson *et al.*, 1992). Several rodent studies support an association between Mn exposure and increased brain GABA concentration (Gwiazda *et al.*, 2002; Reaney *et al.*, 2006). However, other rodent studies suggested that Mn decreases striatal and frontal cortex GABA levels (Seth *et al.*, 1981; Brouillet *et al.*, 1993) or has no effect on GABA levels (Bonilla *et al.*, 1994). Additional findings also suggest that in the absence of extracellular  $\text{Ca}^{2+}$ , Mn induces a long-lasting potentiation of acetylcholine (ACh) release from cardiac parasympathetic nerve terminals following tetanic nerve stimulation (Kita *et al.*, 1981). In combination





**FIGURE 38.2** Photomicrographs of mouse striatal sections with representative tracings of medium spiny neurons (MSNs) from mice treated with saline (control) (A) or  $\text{MnCl}_2$  (100mg/kg, sc) (B). Brain from mouse exposed three times (day 1, 4 and 7) to  $\text{MnCl}_2$  was collected 24 h post last injection. Treatment with Mn induced degeneration of striatal dendritic system, decrease in total number of spines and length of dendrites of MSNs. Tracing and counting are done using a NeuroLucida system at 100 $\times$  under oil immersion (MicroBrightField, VT). Colors indicate the degree of dendritic branching (yellow = 1°, red = 2°, purple = 3°, green = 4°, turquoise = 5°).

with glutamate-gated cation channel activation, e.g., N-methyl-D-aspartate (NMDA) receptor, secondary excitotoxicity mechanisms play an important role in the development of Mn-induced neurodegeneration.

Neurotoxicity of Mn reflects alterations in the integrity of DAergic striatal neurons and DA neurochemistry, including decreased DA transport function and/or striatal DA levels. The striatum is a major recipient structure of neuronal afferents in the basal ganglia. It receives excitatory input from the cortex and DAergic input from substantia nigra and projects to the internal segment of the globus pallidus (Dimova *et al.*, 1993; Saka *et al.*, 2002). Nigrostriatal DAergic neurons appear to be particularly sensitive to Mn-induced toxicity (Sloot and Gramsbergen, 1994; Sloot *et al.*, 1994; Defazio *et al.*, 1996). Intense or prolonged Mn exposure in adulthood causes long-term reductions in striatal DA levels and induces a loss of autoreceptor control over DA release (Autissier *et al.*, 1982; Komura and Sakamoto, 1992). Nigrostriatal DA axons synapse onto striatal medium spiny neurons (MSNs), and these neurons have radially projecting dendrites that are densely studded with spines (Wilson and Groves, 1980). Recent data show the effects of Mn on degeneration of striatal neurons. Representative images of Golgi-impregnated striatal sections with their traced MSNs from control and Mn-exposed animals are presented in Figure 38.2. Images of neurons with NeuroLucida-assisted morphometry show that Mn-induced oxidative damage and neuroinflammation targeted the dendritic system with profound dendrite regression of striatal MSNs. While a single Mn exposure altered the integrity of the dendritic

system and induced significant decrease in spine numbers and total dendritic lengths of MSNs, prolonged Mn exposure led to further reduction in spine numbers and dendritic lengths (Milatovic *et al.*, 2009). In essence, MSNs neurodegeneration could result from loss of spines, removing the pharmacological target for DA-replacement therapy, without overt MSNs death (Stephens *et al.*, 2005; Zaja-Milatovic *et al.*, 2005).

## TOXICITY

Manganese (Mn) is considered to be one of the least toxic of the essential elements (NRC, 2005). There are no reports of acute toxicity of Mn in animals. Therefore, all toxicity studies described here are chronic in nature. A diet can be consumed without any adverse effect when the Mn level is 2000ppm for calves, 3000ppm for sheep, 3000ppm for chickens, 4000ppm for turkeys and 7000ppm for rats. However, decreased growth is observed at 500–3000ppm in swine. These data indicate that pigs are more sensitive to excess Mn than other livestock (NRC, 2005). Mn at a 5000ppm dietary level is lethal to preruminant calves (Puls, 1994). Clinical signs of toxicity include reduced appetite and growth rate, anemia and abdominal discomfort. Excess Mn may be associated with abortions and cystic ovaries. In all domestic animals and poultry, excess dietary Mn is known to cause reduced feed intake, growth rate and lethargy. In dogs, a neurological syndrome of gait



disorders is common and indicative of Mn-induced injury to the extrapyramidal motor system in the brain.

Mn can have a damaging effect on many body organs, including the brain, liver, pancreas and reproductive system. In addition to these well-established neurotoxic effects, Mn has been extensively studied for its reproductive and developmental effects. Evidence obtained from laboratory animals indicates that exposure to high levels of Mn may adversely affect sperm quality (Elbetieha *et al.*, 2001; Ponnappakkam *et al.* 2003a, b), cause decreased testicular weights (Laskey *et al.*, 1982) and impair development of the male reproductive tract. Impaired fertility was observed in male mice exposed to Mn in drinking water for 12 weeks at a daily dose level of 309mg/kg/day, but not at doses  $\leq 154$ mg/kg/day (Elbetieha *et al.*, 2001). Decreased sperm motility and sperm counts were observed in male CD-1 mice after 43 days of exposure to manganese acetate (4.6 to 9.6mg/kg/day). But these doses did not impair the ability of these males to impregnate unexposed females (Ponnappakkam *et al.*, 2003a, b). Szakmary *et al.* (1995) reported that Mn did not result in any reproductive effect in the rabbit when exposed to 11, 22 or 33mg/kg/day on gestation days 6–20. In 13-week dietary studies, no gross or histopathological lesions or organ weight changes were observed in reproductive organs of rats fed up to 618mg Mn/kg/day or mice fed up to 1950mg Mn/kg/day.

Many developmental toxicity studies in animals exposed to Mn have focused on possible effects on reproductive and neurological functions. Animal studies have shown that Mn exposure decreased the growth of reproductive organs (preputial gland, seminal vesicle and testes) (Gray and Laskey, 1980). Testes weights in males were significantly decreased from controls only when Mn was administered in conjunction with an iron-poor diet (Laskey *et al.*, 1982). Studies in neonatal animals have detected structural and neurochemical changes at doses of Mn similar to or slightly above dietary levels (1–10mg Mn/kg/day) (Chandra and Shukla, 1978; Deskin *et al.*, 1980), suggesting that young animals might be more susceptible to Mn than adults. Another study by Dorman *et al.* (2000) also suggested that neonatal rats are at greater risk than adults for Mn-induced neurotoxicity when compared under similar exposure conditions. Their study showed that oral Mn exposure (11 or 22mg/kg/day for 21 days) induced a significant increase in amplitude of the acoustic startle reflex and an increase in striatal DA and 3,4-dihydroxyphenylacetic acid (DOPAC) concentrations in neonates treated with high doses. In a similar study, neonatal rats exposed to Mn (0.31mg Mn/kg/day for 60 days in water) suffered neuronal degeneration and increased brain monoamine oxidase on day 15 and 30 of the study, but did not show any clinical or behavioral signs of neurotoxicity (Chandra and Shukla, 1978). Developmental studies involving the use of laboratory

animals have also detected subtle changes in growth (decreased body weight in animals provided with relatively high doses on Mn). These changes were observed both when the animals were exposed while *in utero* or postpartum. In a developmental rat model of chronic Mn toxicity, administration of Mn in drinking water was associated with increased levels of iron, copper, selenium and calcium in various brain regions. The biochemical mechanisms underlying the interaction between Mn and other minerals are unclear. For further details on Mn-induced reproductive and developmental effects, readers are referred to a recent publication (Milatovic *et al.*, 2011).

Several studies have also addressed gene expression changes in Mn-treated cells and animals and the complex interaction of Mn with other minerals (Baek *et al.*, 2004; HaMai *et al.*, 2006). Mn-induced expression changes were noted in genes involved in inflammation, DNA replication and repair. Recent work in nonhuman primates (Guilarte *et al.*, 2008) detected Mn-induced brain gene expression changes associated mainly with genes affecting apoptosis, protein folding and degradation, inflammation and axonal/vesicular transport.

## DIAGNOSIS

Diagnosis of Mn deficiency and toxicity is based on quantitative determination of Mn in biological specimens (body tissue or fluid). Normal values of Mn in liver and kidney are reported in cattle (2.5–6.0ppm and 1.2–2.0ppm), horses (1.0–6.0ppm and 0.5–2.4ppm), sheep (2.0–4.4ppm and 0.8–2.5ppm), goats (2.0–6.0ppm and 1.0–3.0ppm), pigs (2.30–4.00ppm and 1.30–2.0ppm), dogs (3.0–5.0ppm and 1.2–1.8ppm) and rabbits (1.0–2.0ppm and 2.0–3.0ppm). Mn levels are also reported for blood in cattle (0.07–0.09ppm), sheep (0.02–0.025ppm), dogs (0.02ppm), pigs (0.04ppm) and poultry (0.085–0.091ppm). From human studies it is indicated that neither blood nor urinary Mn levels correlate with any neurological manifestations. Analysis is performed by Flame Atomic Absorption or Furnace Atomic Absorption Spectrometer or Inductively Coupled Plasma (ICP)/ICP-Mass Spectrometer. It is worth mentioning that none of the methods distinguish between different oxidation states of Mn.

## TREATMENT

The very first step in the treatment of Mn poisoning should be to remove the animals from any further exposure by avoiding contaminated feed, water or any other source. Some studies investigating Mn poisoning

suggest that chelation therapy may be considered to reduce the body burden of Mn and to alleviate toxicity symptoms. Chelators bind metal ions in a stable form and the compound chelator plus metal are then excreted by the urinary and/or biliary routes. Ethylene diamine-tetraacetic acid (EDTA) is a polyaminocarboxylic acid that chelates many divalent metals, a property that finds commercial application as a metal sequestrant in food additives. Several studies suggested that EDTA successfully increased Mn excretion in urine and decreased Mn concentration in blood. However, EDTA cannot effectively chelate and remove Mn ions from brain and damaged neurons, and it appears to be of limited therapeutic value for more advanced cases of Mn intoxication.

Since Mn has been shown to catalyze the oxidation of dopamine *in vitro* and the production of dopamine quinone and hydrogen peroxide, it was also suggested that antioxidants may be effective in suppressing Mn toxicity. In addition, interference with oxidation of Mn may affect cellular uptake, elimination of  $Mn^{3+}$  and neurotoxicity. Further investigation of the inhibition of Mn oxidation as a possible mitigation method should be preceded by additional studies to elucidate the role of Mn in its various oxidation states in normal cell metabolism and to determine whether oxidative stress is a primary mechanism for neurotoxicity by Mn exposure.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Acute manganese (Mn) poisoning is rare in animals, but chronic toxicosis can occur as a result of excess dietary exposure. Mn-induced toxicity can affect several body organs involving multiple mechanism of actions. Several studies suggest that oxidative stress, mitochondrial dysfunction and neuroinflammation are underlying mechanisms in Mn-induced vulnerability of dopaminergic (DAergic) neurons. Mediation of any of these mechanisms and control of alterations in biomarkers of oxidative injury, neuroinflammation and synaptodendritic degeneration may provide a therapeutic strategy for the suppression of dysfunctional DAergic transmission and slowing the neurodegenerative process. In addition, multiple mechanisms of Mn action are not sufficiently known and may vary with environmental factors and susceptibilities, including single nucleotide polymorphisms that may alter Mn homeostasis, Mn transport and metabolism. Therefore, further research is required to investigate the direct link between Mn uptake, distribution, accumulation and its downstream target(s), as well as associated clinical manifestations. Importantly, more conclusive studies on mechanisms associated with the extracellular transport

on Mn, mechanistic effects of Mn at the molecular level and its effects on signal transduction pathways, as well as studies on effective diagnosis and treatment, are needed.

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# Mercury

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## INTRODUCTION

Mercury (Hg) is a naturally occurring element that exists in several forms, such as elemental (metallic), inorganic and organic. About 80% of the mercury released into the environment is metallic mercury, and it comes from human activities, such as fossil fuel combustion, mining, smelting and from solid waste incineration, as well as from volcanoes and forest fires. Human activities can lead to mercury levels in the soil as much as 200,000 times higher than natural levels. Metallic mercury in a pure form looks like a shiny-white liquid substance at room temperature. It is commonly used in thermometers, barometers, blood pressure measuring devices, button-cell batteries, electric switches, dental fillings (amalgams), etc. Inorganic mercury compounds, or mercury salts, occur when Hg combines with other elements, such as chlorine, sulfur and oxygen. Most of these compounds are white, except mercuric sulfide or cinnabar ore (i.e., red, which turns black after exposure to light). Some mercury compounds are used as fungicides, while others are used for medicinal purposes, e.g., laxatives, deworming agents, antiseptics and disinfectants. When mercury combines with carbon, it is called organic mercury (organomercurials). Methylmercury, ethylmercury and phenylmercury are a few examples. In the environment and mammalian systems, various forms of mercury are interchangeable. For example, inorganic mercury can be methylated to methylmercury and methylmercury can change to inorganic or elemental mercury. Animals at the top of the food chain tend to bioaccumulate methylmercury in their bodies. Therefore, poisoning by mercury is due to consumption

of meat or grain contaminated with mercury. Poisoning can also result from excessive exposure to inorganic and organic mercury compounds from misuse of mercury-containing products. Much of the information presented in this chapter is from experimental studies conducted in laboratory animals and poisoning incidences in humans, animals and wildlife.

## BACKGROUND

Mercury exists naturally in the environment (soil, water and air), and as a result everyone is exposed to very low levels. Aristotle named it "Quicksilver," because it is a silver-colored liquid. Animal poisoning by mercury is rare because of strict federal, state and local regulations. The most common natural forms of mercury found in the environment are metallic mercury, mercury sulfide, mercuric chloride and methylmercury. Methylmercury is of particular concern because it is bioaccumulated and biomagnified in certain edible freshwater and salt-water fish and marine mammals to levels that are many times greater than levels in the surrounding water. As a result, older and predatory fish living in contaminated water build up levels of mercury in their bodies (especially in the liver, kidneys, brain and muscle). Inorganic mercury does not bioaccumulate in the food chain to any extent. Cultivation of edible mushrooms, where waste as compost material with unsafe levels of mercury is used, can also accumulate high levels of mercury (Bressa *et al.*, 1988). The release of methylmercury into an ocean bay (Minamata) in Japan in the 1950s led to a

massive health disaster, and the clinical syndrome was named Minamata Disease (Tsubaki and Krukuyama, 1977; Tsubaki and Takahashi, 1986). Thousands of people were poisoned, and hundreds developed severe brain damage.

The Food and Drug Administration (FDA) estimates that on average most people are exposed to about 50 ng mercury/kg body weight/day in the food they eat. This level is not enough to cause any harmful effects. A large part of this mercury is in the form of methylmercury and the majority of that comes from eating fish. Fish for food consumption are not allowed to have more than 1 ppm mercury. This level is below a level that can be associated with adverse effects. Foods other than fish that may contain higher levels of mercury include wild animals, birds and mammals (bears) that eat large amounts of contaminated fish (ATSDR, 1999). Meat and/or fat from fish, marine mammals, fish-eating wildlife and birds, and mercury-based fungicide-treated grains have the highest mercury levels. Certain species of commercially available saltwater fish, such as shark, swordfish, kingfish and tilefish, can contain high levels of methylmercury. These are all potential sources of mercury poisoning. In horses, mercury toxicity occurs from wound dressings (blisters) when dimethyl sulfoxide (DMSO) is applied simultaneously, because DMSO enhances the absorption of mercury (Schuh *et al.*, 1988).

## TOXICOKINETICS

Absorption of mercury from oral ingestion depends upon the form of mercury. Metallic mercury is maximally absorbed (about 80%) from the lungs, while very little is absorbed from the GI tract. Once mercury enters the circulation, it is rapidly distributed to other tissues, but more so in the kidneys, where it accumulates. Metallic mercury can stay in the body for weeks to months. Due to its high lipophilicity, metallic mercury can readily cross the blood-brain barrier and placental barrier. When metallic mercury enters the brain, it is readily converted to an inorganic divalent mercury (oxidized by the hydrogen peroxidase-catalase pathway), and it gets trapped there for an extended period. The inorganic divalent cation can, in turn, be reduced to metallic mercury. Most of the absorbed metallic mercury excretes in the urine and feces, some amount passes in the milk, and very little in the exhaled air.

Inorganic mercury compounds (e.g., mercurous chloride and mercuric chloride) are absorbed 10–40% from the GI tract upon ingestion, distributed to different organs and mainly accumulate in the kidneys. In an experimental study, female Sprague-Dawley rats given a single dose of

mercuric chloride (7.4 or 9.2 mg Hg/kg, po) showed 12.6 and 18.9 ppm mercury, respectively, in the kidneys when sacrificed 14 days post-exposure (Lecavalier *et al.*, 1994). Trace amounts were also detected in the liver, brain and serum. These compounds do not readily cross the blood-brain barrier or placental barrier. Inorganic mercury excretes in the urine and feces, and only detectable levels pass through the milk.

Organic mercury, such as methylmercury, is readily absorbed from the GI tract (about 90–95%). After ingestion, the distribution to the blood compartment is complete within 30 h, and the blood level accounts for about 7% of the ingested dose. Circulating methylmercury accumulates predominantly in the red cells where it binds to cysteinyl residues (–SH) on the hemoglobin beta-chain, and is then slowly distributed to other tissues, reaching equilibrium with other tissues at ~4 days. The distribution of methylmercury is similar to that of metallic mercury, i.e., a relatively large amount of mercury can accumulate in the brain and fetus (compared to inorganic mercury) because of its ability to penetrate the blood-brain barrier and placental barrier and its conversion in the brain and fetus to the inorganic divalent cation mercury. Organic mercury excretes in the form of inorganic mercury in the feces over a period of several months. Some organic mercury also excretes in the urine and milk.

Depending upon the route of exposure, dose and single versus repeat exposure, toxicokinetics of mercury can follow a one-compartment or two-compartment model. Studies have shown that repeat or continuous exposure to any form of mercury can result in the accumulation of mercury in the body. In vertebrates, mercury tends to accumulate in the liver and kidney, but it also has a high affinity for the brain and endocrine system. Mercury in the brain may persist long after cessation of short- and long-term exposures. Blood levels of mercury are closely related to its levels in the whole body during the first 3 days following administration. Thereafter, the amount of mercury in the blood declines more rapidly than the whole-body burden. Animal studies show disparity in mercury body burden in terms of gender. In general, females are believed to have higher body burdens of methylmercury at given doses than males. Furthermore, in rodents, females accumulate more mercury in the brain, while males accumulate higher levels of mercury in the kidneys.

Evidence suggests that the metabolism of all forms of mercury is similar for humans and animals. Mercury is metabolized through the oxidation-reduction cycle that takes place in intestinal microflora and after absorption in many tissues and in the red blood cells. Elimination rates for methylmercury appear to vary with species, dose, sex and strain. The elimination half-life in the blood of monkeys receiving inorganic and organic

mercury was found to be 26 days (Vahter *et al.*, 1994). In a study of organs from sled dogs fed methylmercury-laden meat and organs from predatory marine animals, the highest concentration of total mercury was found in the mesenteric lymph nodes, followed by liver and kidneys, indicating that the lymphatic system may play an important role in the transport of mercury to target organs (Hansen and Danscher, 1995). The tissue concentrations of mercury observed in this study were found to be age related, and the results suggest that demethylation takes place in all organs, except the skeletal muscles. Demethylation of methylmercury was found to be lower in the brain than in other organs.

## MECHANISM OF ACTION

Toxicities of the different forms of mercury are related, in part, to its differential accumulation in sensitive tissues. This theory is supported by the observation that mercury rapidly accumulates in the kidneys and specific areas of the brain (the two major target organs). High-affinity binding of the divalent cationic mercury to thiol or sulfhydryl groups of proteins is believed to be a major mechanism involved in the toxicity of mercury. As a result, mercury can cause inactivation of various enzymes, structural proteins, transport proteins and alteration of cell membrane permeability by the formation of mercaptides. In addition, mercury may induce one or more of the following effects: increased oxidative stress, mitochondrial dysfunction, changes in heme metabolism, glutathione depletion, increased permeability of the blood-brain barrier and disruption of microtubule formation, protein synthesis, DNA replication, DNA polymerase activity, calcium homeostasis, synaptic transmission and immune response.

The nervous system is especially sensitive to mercury. The degree of damage depends upon the form of mercury and its dose. Metallic mercury at high doses causes irreparable damage to the brain. In many poisoning incidents, permanent damage to the brain occurred by methylmercury. Since inorganic mercury does not readily cross the blood-brain barrier, it is highly unlikely that inorganic mercury may cause any damage to the brain or nerves. Most of the information concerning neurotoxicity in humans following oral exposure to organic mercury comes from reports describing the effects of ingesting contaminated fish or fungicide-treated grains, or meat from animals fed such grains. Studies conducted in experimental animals strongly indicate that organic mercury is a potent neurotoxicant (Kaur *et al.*, 2007; Ceccatelli *et al.*, 2010; Farina *et al.*, 2011).

Evidence suggests that a single dose of mercuric chloride (0.74 mg/kg) caused disruption of the blood-brain

barrier in rats (Chang and Hartman, 1972). These investigators also administered mercuric chloride to rats at the same dose daily for 11 weeks. Within 2 weeks, there were coagulative or lucid changes in cerebellar granule cells and fragmentation, vacuolation and cytoplasmic lesions in the neurons of dorsal root ganglia. Neurological disturbances consisted of severe ataxia and sensory loss.

Neurotoxic effects seen in the Minamata (Japan) and Iraqi poisonings were associated with neuronal degeneration and glial proliferation in the cortical and cerebellar gray matter and basal ganglia (Al-Saleem, 1976). Derangement of basic developmental processes, such as neuronal migration (Matsumoto *et al.*, 1965; Choi *et al.*, 1978) and neuronal cell division (Sager *et al.*, 1983) were also observed. In the brain, Purkinje, basket, stellate and granule cells are severely affected by methylmercury.

Methylmercury selectively inhibits protein synthesis in the brain (reversibly in neurons from the cerebrum and Purkinje cells; and irreversibly in granule cells of the cerebellum), and this effect usually precedes the appearance of clinical signs. This selective action on the brain may be due to the fact that certain cells are susceptible because they cannot repair damage from methylmercury. Cheung and Verity (1985) identified the most sensitive step in the protein synthesis, i.e., peptide elongation can be affected by the high concentrations of mercury, but the first stage of synthesis associated with tRNA may be the most sensitive. Methylmercury inhibits one or more of the amino acyl tRNA synthetase enzymes. Microtubules are essential for cell division (main component of the mitotic spindle), and methylmercury reacts with the SH groups on tubulin monomers, and thereby disrupts the assembly process. The dissociation process continues, and this leads to depolymerization of the tubule. Excess generation of reactive oxygen species (ROS) and inhibition of antioxidant enzyme appear to be the major mechanisms in methylmercury-induced neurotoxicity (Aschner *et al.*, 2007; Franco *et al.*, 2009; Farina *et al.*, 2009). The most recent findings suggest that methylmercury triggers multiple pathways, which may be activated concomitantly, ensuing cell death by apoptosis (Ceccatelli *et al.*, 2010; Farina *et al.*, 2011).

In all forms, mercury accumulates in the kidneys, and thereby causes greater damage to this organ. The kidney damage appears to be dose dependent, and that means recovery can occur if exposure is at low level. Following entry of the mercuric or methylmercuric ion into the proximal tubular epithelial cells via transport across the brush-border or basolateral membrane, mercury interacts with thiol-containing compounds, such as glutathione and metallothionein. This interaction initially produces alterations in membrane permeability to calcium ions and inhibition of mitochondrial function. Subsequently, by unknown signaling mechanisms, mercury induces the



synthesis of glutathione, glutathione-dependent enzymes, metallothionein and several stress proteins. Finally, epithelial cell damage occurs in the kidney as a result of excess free radical formation and lipid peroxidation, and inhibition of antioxidant enzymes.

## TOXICITY

In general, the toxic effects of mercury depend upon the form of mercury, the dose, duration and route of exposure. Mercury, in all forms, has been found to be toxic to both man and animals. There are many similarities in the toxic effects of the various forms of mercury, but there are also differences. Practically, it is organic mercury which is more toxic and often encountered in poisonings following oral ingestion. The major targets of toxicity to inorganic and organic mercury are the kidneys and the CNS, respectively.

Signs and symptoms associated with short-term exposure to metallic mercury may include nausea, vomiting, diarrhea, increase in blood pressure or heart rate, skin rashes and eye irritation.

Inorganic mercury, if swallowed in large quantities, may cause damage to the kidney, and also in the stomach and intestine, including nausea, diarrhea and ulcers. Animal studies revealed that long-term oral exposure to inorganic mercury salts causes kidney damage, an increase in blood pressure and heart rate, and effects on the stomach. Studies also show that nervous system damage occurs after long-term exposure to high levels of inorganic mercury. Short-term, high-level exposure of laboratory animals to inorganic mercury has been shown to affect the developing fetus and may cause termination of the pregnancy.

Laboratory animals exposed to long-term, high levels of methylmercury or phenylmercury showed damage to the kidneys, stomach and large intestine, changes in blood pressure and heart rate, and adverse effects on the developing fetus, sperm and male reproductive organs, as well as increases in the number of spontaneous abortions and stillbirths.

In livestock animals, clinical signs of mercury poisoning vary greatly. In cattle, toxicity signs include ataxia, neuromuscular incoordination and renal failure, followed by convulsions and a moribund state. Average time from ingestion to death is reported to be about 20 days. Ingestion of phenylmercuric acetate may cause sudden death with massive internal hemorrhage, without other signs of toxicity (Puls, 1994). In horses, signs of acute toxicity include severe gastroenteritis and nephritis. In chronic cases, signs may include neurological dysfunction, laminitis, in addition to renal disease which is

characterized by glycosuria, proteinuria, phosphaturia, reduced urine osmolarity, reduced glomerular filtration rate, azotemia and elevated creatinine and blood urea nitrogen. In sheep, the poisoning is characterized by severe neurological symptoms and tetraplegia. Pigs show incoordination, unstable gait, lameness, recumbency and death.

Some of the toxic effects are described below in detail for each organ/system affected by mercury exposure.

### Nervous system

Adverse effects on the nervous system of animals occur at lower doses than do harmful effects to most other systems of the body. This difference indicates that the nervous system is more sensitive to mercury than are other organs in the body. Animal studies also provide evidence of damage to the nervous system from exposure to methylmercury during development, and findings suggest that the effects worsen with age, even after the exposure stops. The reason for this greater susceptibility is that mercury affects processes unique to the developing nervous system, namely cell migration and cell division (Clarkson, 1987).

Both human epidemiology and experimental animal studies indicate that organic mercury is a potent neurotoxicant. Studies suggest that cats and monkeys are more sensitive than rodents to the neurotoxic effects of mercury (especially methylmercury). In several animal species, the major effects that are seen across the studies include motor disturbances, such as ataxia and tremors, as well as signs of sensory dysfunction, such as impaired vision. The predominant pathological feature is degenerative changes in the cerebellum, which is likely to be the mechanism involved in many of the motor dysfunctions. In a chronic study, cats fed tuna contaminated with methylmercury showed degenerative changes in the cerebellum and the cortex (Chang *et al.*, 1974). Neonatal monkeys exposed to methylmercuric chloride at 0.5mg Hg/kg/day for 28–29 days exhibited stumbling, falling, blindness, crying, temper tantrums and coma. Histopathological analysis revealed diffuse degeneration in the cerebral cortex, cerebellum basal ganglia, thalamus, amygdala and lateral geniculate nuclei (Willes *et al.*, 1978).

Rats acutely intoxicated with methylmercury (19.9mg Hg/kg, oral gavage) showed signs of lethargy and ataxia, which was not accompanied by histopathological changes. Symptoms disappeared within 2–3h. Administration of a single dose of methylmercuric chloride (0.8mg Hg/kg) produced blood–brain barrier dysfunction in rats (Chang and Hartman, 1972) similar to that described for inorganic mercury. Neurotoxic signs observed in rats exposed to methylmercury (4mg Hg/kg/day for 8 days) include muscle spasms, gait disturbances, flailing and hind

limb crossing (Inouye and Murakami, 1975; Fuyuta *et al.*, 1978; Magos *et al.*, 1980, 1985). Histopathological examination of the nervous system of affected rats has shown degeneration of cerebellar granule cells and dorsal root ganglia (Magos *et al.*, 1980, 1985) and degenerative changes in peripheral nerves (Fehling *et al.*, 1975; Miyakawa *et al.*, 1976).

Mice exposed to 1.9 or 9.5 mg Hg/kg/day as methylmercury in the drinking water for 28 weeks exhibited degeneration of Purkinje cells and loss of granular cells in the cerebellum (MacDonald and Harbison, 1977). At higher doses, hind limb paralysis was observed. Neuronal degeneration and microgliosis were observed in the corpus striatum, cerebral cortex, thalamus and hypothalamus, accompanied by hind leg weakness, in mice given 1 or 4 mg Hg/kg/day as methylmercuric chloride by gavage for 60 days (Berthoud *et al.*, 1976). In rabbits given 5.5 mg Hg/kg as methylmercuric acetate for 1–4 days, widespread neuronal degenerative changes in cervical ganglia cells, cerebellum and cerebral cortex have been observed without accompanying behavioral changes (Jacobs *et al.*, 1977).

Following inhalation exposure to metallic mercury vapors, the CNS has been found to be the most sensitive organ in guinea pigs, rats and mice. With increasing concentrations of mercury, damage to CNS becomes irreversible. Rabbits appear to be less sensitive to mercury following inhalation exposure.

## Renal system

Mercury, in all forms, has been shown to cause renal toxicity (structural and functional damage) in humans and animal species that are tested. Renal toxicity has been observed in rats (Fisher 344) and mice (B6C3F<sub>1</sub>) following acute, intermediate and chronic exposures to mercuric chloride (Dieter *et al.*, 1992; NTP, 1993). In a 14-day study, male and female rats were exposed by gavage to 0.93–14.8 mg Hg/kg/day as mercuric chloride for 5 days a week. There was a significant increase in the absolute and relative kidney weights of males beginning at the 1.9 mg Hg/kg/day dose level. An increased incidence of tubular necrosis was observed in rats exposed to at least 3.7 mg Hg/kg/day. Severity was dose dependent. In chronic studies, mercuric chloride produced a variety of pathological changes in kidneys (Carmignani *et al.*, 1992; Hultman and Enestrom, 1992; NTP, 1993). Degenerative effects have been found in the kidneys of animals exposed to moderate to high levels of metallic mercury vapors following acute or subacute exposures (Ashe, 1953). Effects ranging from marked cellular degeneration to tissue destruction and widespread necrosis were observed in rabbits exposed to mercury vapor at a concentration of 28.8 mg/m<sup>3</sup> for 2–3 h. In rats, slight degenerative changes (i.e., dense deposits in tubule cells and

lysosomal inclusions) in the renal tubular epithelium were evident following exposure to 3 mg/m<sup>3</sup> mercury vapor for 3 h/day/5 days a week, for 12–42 weeks (Kishi *et al.*, 1978). Low-level, long-term exposure to mercury (0.1 mg/m<sup>3</sup>) has not been found toxic to the kidneys of rats, rabbits and dogs (Ashe, 1953).

## Cardiovascular system

Mercury has been shown to produce adverse effects on the cardiovascular system. A decrease in heart rate was observed in male rats given two gavage doses of 2 mg Hg/kg as methylmercuric chloride (Arito and Takahashi, 1991). An increase in systolic blood pressure was observed in male rats after daily oral gavage doses of 0.4 mg Hg/kg/day as methylmercuric chloride for 3–4 weeks (Wakita, 1987). This effect began approximately 60 days after initiation of exposure and persisted for at least 9 months.

## GI tract

Ingestion of mercuric chloride is highly irritating to the tissues of the GI tract. Inflammation and necrosis of the glandular stomach were observed in mice that were given oral doses of 59 mg/kg as mercuric chloride 5 days a week for 2 weeks (NTP, 1993). In a 2-year gavage study, an increased incidence of forestomach hyperplasia was observed in male rats exposed to 1.9 or 3.7 mg Hg/kg/day as mercuric chloride compared to the control group. Mice showed ulceration of the glandular stomach after 2 years of dietary exposure to methylmercuric chloride at 0.69 mg Hg/kg/day (Mitsumori *et al.*, 1981, 1990).

## Hematopoietic system

In general, acute mercury toxicity does not produce any characteristic hematological changes. In a chronic study conducted in rats, phenylmercuric acetate given in water at a dose of 4.2 mg Hg/kg/day caused decreases in hemoglobin, hematocrit and RBC counts (Solecki *et al.*, 1991). The anemia observed in this study may have been secondary to blood loss associated with the ulcerative lesions in the large intestine. However, methylmercuric chloride at a low dose (0.1 mg Hg/kg/day for 2 years) given in the diet for 2 years caused no changes in hematological parameters (Verschuuren *et al.*, 1976).

## Other effects

Mercury has been found to have the potential for inducing genotoxicity (Ghosh *et al.*, 1991), carcinogenicity

(Solecki *et al.*, 1991; NTP, 1993), immunotoxicity (Thuvander *et al.*, 1996), and endocrine, reproductive and developmental toxicity (Fuyuta *et al.*, 1979; Castoldi *et al.*, 2008; Liang *et al.*, 2009; Tan *et al.*, 2009; Vitalone *et al.*, 2010; Ni *et al.*, 2011).

## Diagnosis

Presently there are reliable and accurate ways to measure mercury levels in the body, using atomic absorption spectrometer and inductively coupled plasma (ICP) or ICP-mass spectrometer (ICP-MS). Mercury analysis is usually performed on blood, urine, milk, hair, nail, liver and kidney. Mercury in urine is determined to test for exposure to metallic or inorganic mercury, while whole blood or hair values are used to determine exposure to methylmercury. Mercury levels in the blood provide more useful information after recent exposures than after long-term exposures. Levels found in blood, urine and hair may be used together to predict possible health effects that may be caused by the different forms of mercury. The kidney is an ideal specimen for mercury analysis from dead animals.

## TREATMENT

Activated charcoal (1–3g/kg body weight, po) is very effective in reducing further absorption of mercury from the GI tract. Specific treatment of mercury poisoning rests with the use of chelators, along with protein solutions to bind and neutralize mercury compounds. The use of a particular chelator is dependent upon the type of mercury exposure. Among several chelators, dimercaprol (BAL, 3mg/kg, im) has been found to be the most effective against mercury poisoning. However, chelation releases mercury from soft tissues which can be redistributed to the brain. Oral administration of sodium thiosulfate (1g/kg) can assist in eliminating mercury. Animal studies suggest that antioxidants (particularly vitamin E) may be useful for decreasing the toxicity of mercury. Improved chelation and drug therapies for treating acute and chronic mercury poisonings are greatly needed.

## CONCLUSIONS

Toxicity by mercury depends upon the form of mercury, dose, duration and route of exposure. Organic mercury tends to bioaccumulate in the higher food chain, and as a result the maximum concentrations are found in the meat of fish, marine mammals and fish-eating birds and

wildlife. Methylmercury is the most toxic among the mercury species because of its volatility and its ability to pass through biological membranes such as the blood–brain barrier and the placental barrier. The nervous system and kidneys are the two major target organs. Not all forms of mercury cross the blood–brain barrier (e.g., inorganic mercury), but in all forms it accumulates in the kidney and thereby causes damage to this organ. Chelation therapy appears to be the best treatment. Oral administration of activated charcoal is very effective in reducing the further absorption of mercury from the GI tract.

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# Molybdenum

Jeffery O. Hall

## INTRODUCTION

Molybdenum (Mo) is an essential nutrient in plants and animals. Thorough reviews on Mo have been published (Dick, 1956; Underwood, 1977; Ward, 1978; Friberg and Lener, 1986; Mills and Davis, 1987; Rajagopalan, 1988; Nielsen, 1996; Johnson, 1997; NRC, 2006). In plants and microbes, reduction of nitrate to nitrite and nitrogen fixation requires Mo (Williams and daSilva, 2002). Higher animals require Mo for oxygen transfer reactions of aldehyde oxidase, sulfite oxidase and xanthine oxidase, where Mo is bound to a pterin nucleus (Johnson *et al.*, 1980). Although dietary clinical deficiencies have not been reported under natural conditions (Mills and Davis, 1987), deficiency has been produced in animals fed purified Mo deficient diets (Mills and Bremner, 1980; Anke *et al.*, 1985). Functional Mo deficiency has been caused by genetic disorders in humans (Reiss, 2000) and competitive replacement of tungsten for Mo in enzymes (Nell *et al.*, 1980). And, iatrogenic Mo deficiency, resulting in aberrant sulfur-containing amino acid metabolism, has been reported following prolonged total parenteral nutrition (Abumrad *et al.*, 1981).

Mo toxicity is intricately tied to interactions with copper and sulfur. Predominant manifestations of Mo poisoning are associated with secondary copper deficiency, but not all clinical symptoms are alleviated by copper supplementation. The copper-sulfur-Mo interactions are complex and vary greatly in degree of severity among species.

## BACKGROUND

Mo is a transition metal within group VI of the periodic table. It has an atomic number of 42, an atomic weight of 95.95 and has seven different naturally occurring atomic masses from 92 to 100 (Rosman and Taylor, 1998). Mo can occur in a variety of oxidation states that range from (-II) to (VI) (IMOA, 2006), but valence states IV, V and VI are the most common in biological systems (Johnson, 1997). Mo is utilized in the production of oxidation catalysts, pigments, corrosion-resistant steel, smoke suppressants, lubricants, fertilizers and metal alloys. Although uniformly found in nature, the United States has the greatest producible Mo reserves.

Mo is commonly found in low concentrations in most dietary constituents (Rajagopalan, 1988), but excess intake can occur from plants grown on soils naturally high in Mo or from areas contaminated by mining or smelting operations. Naturally high soil and forage molybdenum concentrations have been reported in very localized areas of several states in the western United States, as well as Canada, England, Australia and New Zealand, but likely occurs in other countries as well. In addition, high molybdenum forages have been identified from contaminated areas associated with mining and industrial operations (King *et al.*, 1984). Daily dietary requirements for all species are such that requirements are met, even with low intake.

## PHARMACOKINETICS/ TOXICOKINETICS

### Absorption

Mo absorption differs between monogastrics and ruminants. In monogastrics, Mo absorption occurs from the stomach throughout the intestinal tract (Bell *et al.*, 1964; Miller *et al.*, 1972; Nielsen, 1996). In contrast, ruminant absorption occurs in the intestinal tract, as an extensive delay in peak blood concentration would indicate that rumen absorption does not occur. This mucosal absorption is via an active carrier-mediated process that is also utilized by sulfate (Mason and Cardin, 1977). Absorption is quite efficient, being from 40 to 90% (Friberg and Lener, 1986; Turnlund *et al.*, 1995). But, Mo absorption does not appear to be regulated at the point of mucosal absorption, as increasing Mo concentrations presented to the mucosa result in concomitant increased absorption (Miller *et al.*, 1972; Turnlund *et al.*, 1995).

Dietary constituents can limit Mo absorption. Dietary sulfate present at the point of absorption can competitively inhibit Mo uptake (Mason and Cardin, 1977). Furthermore, in the presence of sulfur/sulfates, the reductive rumen metabolism results in di-, tri- and tetra-thiomolybdates, which can then bind copper and form a non-absorbable cupric thiomolybdate complex (Dick, 1956; Price *et al.*, 1987; Gooneratne *et al.*, 1989).

### Distribution

Mo is widely distributed in tissues, but has highest concentrations in the liver, kidney and bone (Schroeder *et al.*, 1970; Friberg and Lener, 1986). In light of the essential nature of Mo, it is somewhat unusual that very little tissue retention/reserve is maintained. Post-absorptive circulation occurs by transport bound to the red blood cell proteins or as free ionic molybdate (Allway *et al.*, 1968; Versieck *et al.*, 1981). But, absorbed or systemically produced thiomolybdates can bind copper and result in circulating copper–thiomolybdate complexes which are not biologically available for tissue utilization.

### Elimination

Mo is eliminated from the body fairly rapidly, with little, and only short-term, tissue retention. Although urinary is the primary route of elimination, biliary elimination also occurs (Friberg and Lener, 1986; Vyskocil and Viau, 1999; NRC, 2006) and likely is the primary route of elimination in ruminants (Grace and Suttle, 1979; Pott *et al.*, 1999). Urinary elimination is concentration dependent,

resulting in relatively rapid elimination even with very large exposures. In lactating animals, Mo is excreted in the milk with content being dependent on the concentration being ingested (Archibald, 1951; Anke *et al.*, 1985). Thus, exposure can be approximated by analysis of urine or milk for Mo content across time and extrapolating back to the time of exposure (Lesperance *et al.*, 1985). Just as sulfate can inhibit the absorption of Mo, it can also compete for reabsorption sites in the renal tubules and enhance the rate of elimination (Friberg and Lener, 1986).

## MECHANISM OF ACTION

The mechanism by which Mo is active in biologic systems is through its redox activity in functional molybdo-enzymes (Mills and Davis, 1987). The readily changeable oxidation states of Mo lend it to functional utilization in these types of reactions.

The primary mechanisms by which Mo is toxic are directly tied to its interactions with sulfur and copper. These interactions result in functional or overt copper deficiency. But, these interactions differ significantly among species, with ruminants being much more susceptible than monogastrics. The reducing environment of the rumen converts sulfate or sulfur from sulfur-containing amino acids to sulfide, which then forms mono-, di-, tri- and tetra-thiomolybdates (Price *et al.*, 1987; Spears, 2003). Thiomolybdates binding of copper in the digestive tract prevents absorption of ingested copper, while systemic binding renders it non-bioavailable for tissue utilization (Gooneratne *et al.*, 1989; Suttle, 1991). These cupric-thiomolybdate complexes also result in enhanced copper excretion (Howell and Gooneratne, 1987). Price *et al.* (1987) found that the ruminal binding was predominantly via tri- and tetra-thiomolybdates, while systemic effects were predominantly via di- and tri-thiomolybdates. In practical means, the thiomolybdates serve as effective chelators of copper, preventing copper absorption and depleting functional body stores. As the ruminal microbial populations can differ significantly among ruminant species, the relative sensitivity among species could be related to the overall conversion to thiomolybdates or the relative abundances of the mono-, di-, tri- and tetra-thiomolybdates produced.

Most of the clinical syndromes of Mo poisoning can be tied to deficiencies in copper-containing enzyme systems (NRC, 2006). Although most clinical effects of Mo poisoning are reversed by supplementation of copper, Mo may have some direct toxic effects. It is possible that permanent tissue damage, caused by severe copper depletion, results in non-response to copper supplementation in clinically affected animals. The exact mechanisms of

non-copper responsive toxic effects of Mo are poorly defined or investigated, but it has been observed that high Mo concentrations can inhibit the *in vivo* activity of biological enzymes, such as succinic acid oxidase, sulfide oxidase, glutaminase, cholinesterase and cytochrome oxidase (Venugopal and Luckey, 1978).

## TOXICITY

Both acute and chronic toxicity of Mo varies greatly among species. Monogastric animals are much less sensitive than ruminants, due to the rumen metabolism of sulfur and formation of thiomolybdates. The relative tolerance to Mo has been ranked: horses > pigs > rats > rabbits > guinea pigs > sheep > cattle (Venugopal and Luckey, 1978), but more recent literature suggests horses may be more sensitive (Ladefoged and Sturup, 1995). In total, the toxicity of Mo needs to be evaluated with consideration of dietary sulfur/sulfates and copper. This is with knowledge that sulfates competitively inhibit uptake from the intestinal tract, but in ruminants results in the formation of thiomolybdates which will enhance the toxic effects on copper status.

Natural toxic effects of Mo are primarily via ingestion, but toxicity has been demonstrated by both inhalation and injection in laboratory rodents. Intraperitoneal LD<sub>50</sub> of Mo in rats ranges from 99 to 22.8 mg/kg body weight (Venugopal and Luckey, 1978), with similar lethal doses in mice and guinea pigs. Chronic inflammatory lesions and hyaline degeneration within the respiratory tree was induced by Mo trioxide exposure of 10–100 mg/m<sup>3</sup> 6 h per day, 5 days a week (Chan *et al.*, 1998). Increased incidence of respiratory adenomas was also seen in the rats and mice.

Concentrations of Mo required to produce acute poisoning orally differ significantly among species. For cats, rabbits and guinea pigs, the oral LD<sub>100</sub> for Mo is 1310, 1020 and 1200 mg/kg body weight, while the oral LD<sub>50</sub> for rats is 125–370 mg/kg body weight (Venugopal and Luckey, 1978). Little acute toxicity data are available for domestic animals, but cattle have been acutely poisoned with feed containing 7400 mg Mo/kg diet (group average intake of 31 mg Mo/kg body weight/day) and Mo was acutely lethal in sheep at 132–137 mg Mo/kg body weight/day for 2–3 days (Swan *et al.*, 1998).

Clinical signs and pathologic lesions in acutely poisoned animals differ from those seen with more chronic poisonings. Acutely poisoned cattle and sheep developed feed withdrawal, lethargy, weakness, hind limb ataxia that progressed to the front limbs and recumbency (Swan *et al.*, 1998). The cattle also had profuse salivation, ocular discharge and mucoid feces. Hydropic hepatocellular degeneration/necrosis and hydropic degeneration/

necrosis of the proximal and distal renal tubules was observed in both cattle and sheep.

Subacute to chronic toxicity of Mo is just as species dependent as acute poisoning. Oral lethal doses of Mo administered chronically to laboratory animals (rats, mice, guinea pigs and rabbits) range from 60 to 333 mg Mo/kg body weight/day, while cattle are poisoned with as little as 3 mg Mo/kg body weight/day (NRC, 2006). Literature reports of toxic dietary Mo content range from 10 to 1200 ppm for rats, 300 to 8000 ppm for poultry, 200 to 4000 ppm for rabbits, 1000 to 8000 ppm for Guinea pigs, 2.5 to 20 ppm for sheep and 2 to 400 ppm for cattle (Pitt, 1976). Ruminants commonly succumb to molybdenosis when Mo intake is greater than 20 ppm in the diet. However, due to the intrinsic nature of the Mo–copper–sulfur interactions, chronic Mo poisoning in ruminants can be divided into three classes: dietary Mo greater than 20 ppm, low Cu:Mo ratio (<2:1) and high dietary sulfur with normal copper and Mo (Ward, 1978). The desired Cu:Mo ratio in ruminants is between 6:1 and 10:1 (Thompson *et al.*, 1991). Thus, Mo toxicosis can occur at much low concentrations in association with copper deficient forages than those with diets adequate in copper.

Most clinical signs of chronic Mo poisoning are associated with induced copper deficiency. Commonly, the first recognized clinical sign of chronic Mo poisoning is severe diarrhea (Dick, 1956; Pitt, 1976; Underwood, 1977; Ward, 1978; Friberg and Lener, 1986; Mills and Davis, 1987; Rajagopalan, 1988; Nielsen, 1996; Johnson, 1997; Coppock and Dziwenka, 2004; NRC, 2006). “Teart” is used to refer to soil or plants that contain unusually high amounts of Mo, thus the term teart scours is commonly used to describe the diarrhea associated with excessive Mo intake. Although the exact mechanism is not well defined, copper supplementation alleviates this clinical sign. Other common clinical signs of chronic Mo poisoning include poor body weight gain, weight loss, anemia, decreased milk production, achromotrichia, alopecia, limb deformities, bone fractures, periostosis, lameness, lack of libido and ataxia. Abortions have been reported in horses.

Pathologic alterations of chronic Mo poisoning are not specifically diagnostic. These lesions are secondary to induced copper deficiency and include emaciation, periostosis and epiphyseal plate growth abnormalities. Because of the nature of chronic Mo poisoning, primary copper deficiency must always be ruled out when these lesions are identified.

## TREATMENT

The two primary mechanisms of treating Mo toxicosis involve removal from the source of high Mo and

copper supplementation, but administration of sulfate to monogastrics will enhance elimination rates. Administration of sulfates to ruminants would not be recommended, as ruminal reduction to sulfide could potentially result in greater thiomolybdate concentrations and worsen the clinical disease. With rapid clearance from the body, removal from the high Mo source will quickly remove excess Mo from the body. However, permanent damage may be present that results in sustained animal deaths for weeks to months post-exposure (Swan *et al.*, 1998). Supplementation of copper in Mo-poisoned animals must be done with care, especially in sheep, to prevent excessive copper accumulation and subsequent copper toxicosis.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

The known mechanisms of Mo poisoning are a complex interaction with sulfur and copper that differ significantly across animal species. But, some clinical effects of Mo poisoning have not been related to absolute or functional copper deficits, as replenishment of copper does not result in cessation of clinical effects. These clinical effects of Mo poisoning need further investigation to delineate the mechanism by which Mo produces adverse animal health.

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# Selenium

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## INTRODUCTION

Selenium is an essential nutrient that has a relatively narrow window between ingested amounts that result in deficiencies and those that cause toxicoses. Historically, occurrences of livestock disease that mimic clinical presentation of chronic selenium poisoning were recorded in the 13th century (Martin, 1973). Marco Polo wrote of such cases in western China in 1295. In 1560, Father Simon Pedro described human cases of presumably chronic selenosis in Columbia (Benavides and Mojica, 1965). The first documented record of selenium poisoning in livestock was reported in 1860 by a U.S. Army surgeon (Martin, 1973). T.W. Madison described a fatal disease of horses that grazed near Fort Randall, South Dakota. It also has been speculated that the horse illness that slowed General Custer's Cavalry relief may have been due to selenium, but chronic selenosis generally takes weeks to develop.

Selenium deficiency had been historically linked to a variety of clinical effects. Since 1949, vitamin E, cysteine and a "factor 3" were known to protect rats from fatal liver necrosis (Schwarz and Foltz, 1957). When rats were fed torula, a brewer's yeast, they developed liver necrosis that could be avoided by use of baker's yeast (*Saccharomyces* sp.). It was postulated that an essential nutrient, "factor 3," was deficient in torula. After much research, the active, preventive element present in "factor 3" was identified as selenium. Several metabolic diseases of previously unknown origin were later found to relate to selenium deficiency, including "white muscle disease" (WMD) in calves and lambs (Muth *et al.*, 1958; Godwin and Fraser, 1966), hepatitis dietetica in pigs (Eggert *et al.*, 1957), exudative diathesis in poultry

(Patterson *et al.*, 1957) and pancreatic degeneration in poultry (Thompson and Scott, 1969).

Since its first discovery as an essential nutrient, selenium has been found to act in numerous body systems. In 1973, Se was identified as an essential component of glutathione peroxidase (GSH) enzyme (Flohe *et al.*, 1973; Rotruck *et al.*, 1973). Selenium was shown to be essential in humans when added dietary selenium prevented a cardiomyopathy known as "Keshan disease" (Chen, 1986). Selenium supplementation may also be protective against certain types of cancer (Combs, 1997), cardiovascular disease (Duthie *et al.*, 1989) and viral infections (Schrauzer, 1994; Levander, 2000). Because of the essential nature of selenium, poisoning cases from both natural plant accumulations of selenium and nutritional overdoses are encountered.

The identified essential functions of selenium are still increasing. The most notable is GSH, where selenocysteine is a required component of the enzyme system (Brown and Arthur, 2001). Reduced GSH is the primary physiologic first defense against free radical damage to tissues, helping to maintain functional membrane integrity. Several subclasses of GSH are now recognized (Cohen and Avissar, 1994; Sunde, 1994). To date over 30 selenoproteins have been identified, many of which have vital enzymatic functions (Tiwary, 2004). Thioredoxin reductase I, II and III (Brown and Arthur, 2001), 5' triiodothyronine deiodinase (Arthur and Beckett, 1994) and "selenoprotein" are also selenium dependent. In addition, selenium plays several roles in normal immune function, reproductive function, hepatic biotransformation reactions, neurotransmitter turnover and anti-carcinogenic functions.

## BACKGROUND

Selenium is a member of the non-metallic elements within group VIA of the periodic table. It has an atomic number of 34, an atomic weight of 78.96, and has six different naturally occurring stable isotopic masses from 74 to 82 (Rosman and Taylor, 1997). Selenium has four natural oxidation states:  $-2$  (selenides),  $0$  (elemental),  $+4$  (selenites) and  $+6$  (selenates) (Barceloux, 1999). Selenium was first identified in 1817 by Jons Jakob Berzelius, a Swedish chemist, who investigated worker illnesses in a sulfuric acid plant at Gripsholm, Sweden (Fredga, 1972). He named this element after "Selene," the green moon goddess.

Many areas within the Northern Great Plains of the United States, such as the Dakotas, Wyoming, Montana, Nebraska and Kansas, have high soil selenium content (4–5 ppm Selenium or more), resulting in high plant uptake and subsequent Se toxicosis in herbivores (Rosenfeld and Beath, 1964). High soil selenium also occurs in alkaline soils of some localities in Algeria, Argentina, Australia, Bulgaria, Canada, China, Columbia, Ireland, Israel, Mexico, Morocco, New Zealand, South Africa, the former Soviet Union, Spain and Venezuela (NRC, 1983). However, total soil selenium is not the best indicator of potential selenium poisonings, as Hawaii and Puerto Rico have areas of high soil selenium that is not available to the plants due to the acidic soil types, which result in lowered water soluble, bioavailable selenium for plant uptake (Lakin, 1961).

Inorganic forms of selenium are the primary form in soil. Only the water soluble forms are readily available for plant uptake, with the greatest absorption being in the form of selenate via the sulfate transporter. Elemental selenium and precipitated metal-selenides are not bioavailable for plant uptake. Some "indicator plants" or "obligatory selenium accumulator plants" can accumulate several thousand ppm selenium and are often found in selenium-rich areas, since they require high selenium for growth (Rosenfeld and Beath, 1964). These plants include genera such as *Astragalus* (milk vetch), *Xylorhiza*, *Machaeranthera* (woody aster), *Haplopappus* (golden weed) – formerly known as *Oenopsis* and *Stanleya* (prince's plume). Selenium content as high as 14,990 ppm have been reported for a sample of *Astragalus racemosus* (Beath, 1937). Although these indicator plants have poor palatability, during times of limited forage, they are eaten. Secondary or facultative accumulating plants can survive with high selenium content, but do not require it for growth. These plants are often more palatable than the indicator plants and include *Aster*, *Atriplex* (salt bush), *Castilleja* (paintbrush), *Gutierrezia* (snakeweed), *Grindelia* (gumweeds), *Sideranthus* (ironweed), *Eurotia* (winter fat), *Mentzelia*, *Machaeranthera* and *Gyria* sp., as well as some crop plants such as western wheat grass, barley, wheat, alfalfa, onions and Swiss chard (Beath *et al.*, 1935; Williams and Byers, 1936).

Most of the selenium in non-indicator plants and other biological matrices is in an organic form, but small amounts of inorganic selenate and selenite can also be present. The vast majority of plant selenium, especially in non-indicator plants, is in the form of selenomethionine, but selenocysteine and a variety of other seleno-amino acid derivatives can also be found (Peterson and Butler, 1962; Olson *et al.*, 1970; Whanger, 2002). In contrast, the majority of selenium in indicator plants, such as *Astragalus*, is a water soluble Se-methyl-selenocysteine (Shrift, 1973), but can also have selenocystathionine (Lewis, 1976). Garlic was found to contain significant selenomethionine, as well as glutamyl-Se-methyl-selenocysteine and possibly gamma-glutamyl-selenomethionine (Kotrebai *et al.*, 1999). The non-protein associated selenium compounds may be a protective mechanism of the plants to prevent excessive replacement of methionine or other sulfur-containing amino acids with seleno-amino acids in plant proteins resulting in loss of disulfide bonds, misfolding and altered protein properties (Peterson and Butler, 1962). Some microbial populations, as well as plants, can reduce selenium to volatile chemical forms (Shrift, 1973).

## PHARMACOKINETICS/ TOXICOKINETICS

### Absorption

The majority of ingested selenium compounds are absorbed from the duodenum, with lesser amounts in the jejunum and ileum (Wright and Bell, 1966; Whanger *et al.*, 1976). Little to no absorption reportedly occurs from the stomach and rumen. However, one report suggests that minimal absorption of selenomethionine occurs through the rumen wall and into the blood (Hidiroglou and Jenkins, 1973).

The chemical form of selenium greatly impacts the overall absorption. Selenite absorption is via passive diffusion through the brush border membranes (Vendeland *et al.*, 1992, 1994). In contrast, selenate has little affinity for the brush-border membranes. Selenate is absorbed via a sodium co-transport system that is also utilized by sulfate (Wolffram *et al.*, 1988). Selenium in the form of seleno-amino acids, selenomethionine and selenocysteine are absorbed through active amino acid transport mechanisms and are more bioavailable than selenite or selenate (McConnell and Cho, 1967; Ammerman and Miller, 1974; Vendeland *et al.*, 1994). The selenium status did not affect overall absorption, indicating that absorption was not under homeostatic regulation.

In monogastrics, the relative selenium absorption is greater than in ruminants, ranging from 45 to 95%

(Thomson and Stewart, 1974; Furchner *et al.*, 1975; Bopp *et al.*, 1982). And organic forms of selenium are better absorbed (Robinson *et al.*, 1978). In ruminants, the relative absorption ranges from 29 to 50% (Wright and Bell, 1966; Suttle and Jones, 1989). The decreased absorption in ruminants is due to microbial reduction of selenium forms in the rumen to selenides and elemental selenium which are not bioavailable (Cousins and Cairney, 1961; Whanger *et al.*, 1968; Peter *et al.*, 1982). This reduction in bioavailability is generally exacerbated by high carbohydrate diets, but can be altered by differing rumen microbial populations (Hudman and Glenn, 1984; Koenig *et al.*, 1997). Some rumen microbes more efficiently reduce selenium, while others effectively incorporate it into selenium-containing amino acids. The incorporation of selenium into microbial proteins, as well as systemic absorption, can be competitively inhibited by natural methionine and cysteine (Serra *et al.*, 1996).

## Distribution

Tissue distribution is dependent on the chemical form of selenium absorbed. Selenium is generally utilized for synthesis of selenoproteins, incorporated into tissue proteins or eliminated. Selenomethionine can be non-specifically incorporated into tissue proteins in place of methionine (Awadeh *et al.*, 1998), but selenocysteine is not (Burk *et al.*, 2001), with highest incorporation occurring in tissues with high rates of protein synthesis (Hansson and Jacobsson, 1966). The non-specific incorporation of selenomethionine effectively serves as a pool of selenium reserve with a long biological half-life (Schroeder and Mitchener, 1972a). Highest total selenium content is typically found in the kidney and liver, with lesser amounts in all other tissues (Muth *et al.*, 1967; Levander, 1987; Echevarria *et al.*, 1988; Davidson and Kennedy, 1993). Both specific and non-specific selenium incorporation into proteins was greater in selenium-deficient animals.

Time to peak tissue concentrations is tissue dependent. Peak selenium content of blood, liver, muscle, kidney, spleen and lung was reached within 24h after an injection of  $^{75}\text{Se}$  as selenite (Muth *et al.*, 1967). In contrast, brain, thymus and reproductive organs do not reach maximal content until much later (Brown and Burk, 1973; McConnell *et al.*, 1979; Smith *et al.*, 1979; Behne *et al.*, 1988).

Selenium is efficiently transferred across the placenta into feti during gestation. The overall maternal selenium content is positively correlated with fetal and newborn selenium status (McConnell and Roth, 1964). Although they get the vast majority of selenium *in utero*, newborns do get minimal amounts of selenium from milk, with much higher content in colostrums than in milk later in lactation.

## Metabolism

Selenite is metabolized in red blood cells to hydrogen selenide (Gasiewicz and Smith, 1978). Sequential methylation reactions result in the formation of monomethylselenide, dimethylselenide and trimethylselenide (Kajander *et al.*, 1991; Itoh and Suzuki, 1997). These reactions utilize *S*-adenosylmethionine for methyl groups which are transferred by methyltransferases (Kajander *et al.*, 1991). These sequential reactions can deplete available *S*-adenosylmethionine, which would limit the degree of methylation. In rats given selenomethionine, trimethylselenide occurred in the urine more rapidly than in rats given sodium selenite or selenocysteine, indicating that selenomethionine may be converted to methylselenol which is easily further methylated.

Selenomethionine is metabolized by demethylation to selenocysteine. This set of pathways is similar to the metabolism of methionine. The selenocysteine is then metabolized by selenocysteine-beta-lyase in the liver and kidney to alanine and selenide (Soda *et al.*, 1987).

## Elimination

Selenium is primarily excreted in the urine and feces, but the form and extent of elimination by different routes are dose and species dependent. In monogastric animals, urinary elimination predominates, irrespective of the route of exposure (Leng *et al.*, 2000), with less than 10% recovered in feces (Burk *et al.*, 1972). Some literature suggests that urinary eliminated selenium is predominantly metabolites of selenium, with trimethylselenide predominating at higher doses (McConnell and Roth, 1966; Palmer *et al.*, 1969; Zeisel *et al.*, 1987; Itoh and Suzuki, 1997), but monomethylselenide is more abundant at lower doses. Human elimination is tri-exponential for selenite and selenomethionine (Alexander *et al.*, 1987). The terminal elimination phase was 8–20 and 230 days for selenite and selenomethionine, respectively. Overall selenium retention and maintenance of adequate selenoenzymes are for a much longer time period in animals supplemented with selenomethionine than selenite. Elimination rate is dose dependent, with half-lives of 19.5 and 1.2 days with selenite of 0.1 and 1.0ppm in the diet, respectively (Burk *et al.*, 1972). Due to non-specific protein incorporation of selenomethionine, urinary and fecal recovery after dosing was less than 30% of that for equal selenium doses from selenite or selenate (Thomson, 1998).

The literature suggests that the predominant selenium elimination in ruminants is fecal when ingested, but urinary with parenteral administration or in non-ruminating young animals. This is actually an error in terminology, as the fecal loss of selenium is primarily in the forms of



elemental selenium and precipitated selenides from ruminal reduction (Langlands *et al.*, 1986). Thus, this selenium is just non-absorbed material and not truly being eliminated from the central compartment. However, a small amount of metabolized selenium excesses is excreted in the bile (Cousins and Cairney, 1961). The selenium metabolites eliminated in the urine follow a similar pattern to that seen with monogastrics. Urinary elimination is predominant with parenteral administrations and in non-ruminating young animals (NRC, 1983).

Renal selenium elimination is dependent of glomerular filtration and degree of reabsorption. Increasing renal fluid absorption did not increase the selenium content in urine, indicating a tubular reabsorptive process (Oster and Prellwitz, 1990). Thus, dehydration or renal insufficiency would decrease rates of elimination. Excretion and renal clearance rates correlate with creatinine, indicating glomerular filtration is the mechanism of elimination.

Some selenium is eliminated via respired air, but the relative importance of this route is dose dependent. At normal intake, only about 10% or less is eliminated from the respiratory tract (Burk *et al.*, 1972), but as dose increases the percent eliminated in respired air increases (Jacobsson, 1966; McConnell and Roth, 1966). Dimethylselenide and dimethyldiselenide are the predominant forms eliminated in respired air at toxic doses. Dimethylselenide predominates when mice were dosed with selenite or selenocysteine, while dimethyldiselenide is most abundant when rats were dosed with selenomethionine. Respiratory elimination is primary when renal elimination thresholds are maximized, which results in most respiratory elimination occurring in a short-time period soon after exposure to toxic doses (McConnell and Roth, 1966; Tiwary *et al.*, 2005).

## MECHANISM OF ACTION

Although much research has been conducted with regard to selenium poisoning, the exact mechanism of the toxic effects in the body are still not clear. With acute poisoning, one theory is the depletion of intermediate substrates, such as glutathione and S-adenosylmethionine, which disturbs their respective enzyme activities (Vernie *et al.*, 1978). Another potentially interactive theory is the production of free radicals by reaction of selenium with thiols, causing subsequent oxidative tissue damage (Hoffman, 2002; Kaur *et al.*, 2003; Balogh *et al.*, 2004). A third theory is the incorporation of selenium compounds in place of sulfur, such as in proteins, in which it disrupts normal cellular functions (Raisbeck, 2000). This is an especially likely mechanism for the hair and hoof lesions of chronic selenium poisoning, with the loss

of disulfide bridges which provides structural integrity to these tissues. This would also apply to inhibition of DNA methylation by S-adenosylmethionine or indirect inhibition by increased S-adenosyl homocysteine content (Hoffman, 1977). And, it is possible that each of these proposed mechanisms is valid with respect to specific chemical forms of selenium. It has been observed that tissues requiring selenium to prevent deficiency-associated disease (immune system, reproductive organs and muscular tissues) also tend to accumulate more selenium with excess exposure and are tissues of toxic effects.

## TOXICITY

Selenium poisoning cases generally fall into three types of exposure history. The first is from ingestion of selenium in plants that have accumulated it from naturally seleniferous soils. The second is from accidental overdoses by injection or errors in feed mixing. And the third is from environmental contamination, which often results in exposure from plant accumulation and or contaminated waters. With each of these types of poisonings, one may see acute, subacute or chronic selenium poisoning, depending upon the daily exposure rate. However, one must understand that an animal's age plays a role in susceptibility to selenium poisoning, as young animals are less tolerant than adults (Raisbeck, 2000).

Doses for acute selenium toxicosis vary significantly across species, but also by route of exposure, with injectable routes of exposure being more toxic than oral. Acute oral selenium poisoning occurs with sudden exposure ranging from 2.2mg/kg (Rosenfeld and Beath, 1964) to greater than 20mg/kg body weight (Miller and Williams, 1940; Mahan and Moxon, 1984) across species. The relative oral acute toxicity of selenium containing compounds is dependent on their solubility, with poorly soluble selenides and elemental selenium being much less toxic than soluble selenates, selenites and organic selenium (NRC, 2005). Minimum lethal dose for rabbits, rats, dogs and cats is 1.5–3mg/kg body weight (NRC, 1983). The LD<sub>50</sub> for oral selenite has been estimated to be 1.9–8.3mg/kg body weight in ruminants (Grace, 1994), but other references suggest it to be 9–20mg/kg body weight (Puls, 1994). In poultry, the acute oral LD<sub>50</sub> of selenium is 33mg/kg body weight. Injectable selenium is more acutely toxic than oral, with intramuscular LD<sub>50</sub> of 0.5mg/kg in lambs (Caravaggi *et al.*, 1970). Subcutaneous LD<sub>50</sub> of selenium is 1 mg/kg in lambs and 1.9mg/kg in adult cattle (Grace, 1994). In 2009, 21 horses from Venezuela that were in Florida for an international polo match died from selenium overdose (Florida, 2009; Desta *et al.*, 2011). The selenium was misformulated in

a compounded intravenous vitamin-mineral product, but the exact amount of the overdose has yet to be published. The horses developed clinical signs within hours of the administration and died within 3 to 18 hours.

Clinical manifestation of acute selenium poisoning begins as early as 8–10h, but can be delayed for up to 36h (Franke and Moxon, 1936; NRC, 1983; Raisbeck, 2000; Tiwary *et al.*, 2006). Early in the clinical syndrome, one can detect the garlicky smell of dimethylselenide on the breath. Clinical signs that follow include respiratory distress, restlessness or lethargy, head down, droopy ears, anorexia, gaunt appearance, salivation, watery diarrhea, fever, sweating, tachycardia, teeth grinding, stilted gait, tetanic spasms and/or death. Clinical signs tend to progress quickly after they are first observed. Gross and histologic lesions include systemic congestion, pulmonary edema and petechial hemorrhages in and on the myocardium.

“Blind staggers” has historically been associated with subacute to chronic selenium. However, this association was due to its occurrence in known seleniferous areas. The areas with seleniferous soils also tend to have highly alkaline soils with high potential for excessive sulfur exposure. It has been stated that blind staggers cannot be reproduced with pure selenium compounds alone and likely involves other factors, such as alkaloid poisoning, starvation or polioencephalomalasia (O’Toole and Raisbeck, 1995). However, one can still find references that tie it to selenium (Underwood and Suttle, 1999; NRC, 2005).

Chronic selenosis, often referred to as “alkali disease,” is the result of long-term ingestion of seleniferous forages (NRC, 1983, 2005; Raisbeck, 2000). High selenium intake is generally for greater than 30 days and, due to plant selenium content, is usually associated with facultative accumulators, not indicator plants, although chronic selenosis can also be reproduced by long-term feeding of high inorganic selenium (Kaur *et al.*, 2003). Calves were chronically poisoned with selenite at 0.25mg/kg body weight daily for 16 weeks. In a similar study in yearlings, selenium as selenite at 0.8mg/kg/day and as selenomethionine at 0.28mg/kg/day resulted in alkali disease (O’Toole and Raisbeck, 1995). However, other studies did not produce alkali disease with selenium doses as high as 11.9mg/kg of diet in feeders or 118mg/kg body weight daily for 128 days in dairy cows (Ellis *et al.*, 1997; Lawler *et al.*, 2004). Differences in susceptibility to chronic selenium poisoning may be a product of historical exposure, variability in rumen microbial population and/or age-associated susceptibility. As stated previously, certain microbes can reduce selenium to non-bioavailable forms, resulting in decreased systemic absorption. Pigs develop chronic selenosis with exposure to selenium as low as 8mg/kg of diet (Goehring *et al.*, 1984; Mahan and Magee, 1991; Stowe and Herdt, 1992). And horses exposed to 20mg Se/kg DM for 3 weeks developed lesions (Stowe and Herdt, 1992).

Clinical signs of chronic selenosis include depression, weakness, emaciation, anemia, hair loss, anorexia, diarrhea, weight loss, lameness and death (Rosenfeld and Beath, 1964; O’Toole and Raisbeck, 1995; Underwood and Suttle, 1999; Raisbeck, 2000). Hoof wall abnormalities are frequently identified in cattle, horses and pigs, and include swelling of the coronary band, hoof deformities and/or separation and sloughing of the hoof wall. Hair loss from the base of the tail and switch in cattle, horses and mules is sometimes referred to as “bobtail disease.” Interestingly, sheep do not develop the alopecia or hoof lesions that are seen in cattle, but they have decreased wool growth rates. In pigs, goats and horses, there may be a general alopecia (Franke, 1934). Pigs also develop neurologic signs of paralysis (Goehring *et al.*, 1984).

Pathologic lesions of chronic selenium poisoning are generally related to hoof lesions and to the effects of starvation (Raisbeck, 2000). Lesions of nephritis, hepatic cirrhosis and myocardial necrosis can be expected. In pigs, bilateral malacia of the gray matter in the spinal cord can be seen.

Reproductive abnormalities are seen (Davis and Hall, 2011) in several species when excessive selenium is ingested. Field reports indicate that reproductive performance can be reduced without the other typical signs of alkali disease at 5–10ppm Se in diet (Olson *et al.*, 1970), but direct experimental evidence is lacking (Raisbeck, 2000). Decreased conception rate and an increased fetal resorption rate in cattle, sheep and horses were observed when they were fed natural diets containing 20–50mg Se/kg diet (Harr and Muth, 1972). Both rats exposed to 3ppm Se as seleniferous wheat (Munsell *et al.*, 1936) and mice exposed to 3ppm selenate in their drinking water (Schroeder and Mitchener, 1972b) had abnormally low rates of conception. Both deficiencies and excesses of selenium have been shown to adversely affect spermatogenesis, motility and viability in rodent models (Kaur and Bansal, 2005; Shalini and Bansal 2007, 2005; Boitani and Puglisi, 2008). If other species have similar effects, this could negatively affect herd reproductive performance. In addition, it must be noted that Se accumulates in the fetus at the expense of the dam (Puls, 1994). Thus, higher accumulation of selenium in the fetus may result in abortions, stillbirths or weak/lethargic calves. Yeager *et al.* (1998) reported a 7% abortion rate in a beef herd where cows had selenium concentrations suggestive of chronic selenosis and fetal hepatic selenium concentrations suggestive of toxicosis, but in a dosing study they only had one weak calf born that died shortly after birth. Although teratogenic effects of selenium poisoning are well documented in avian species, historical reports are all that are available for horses and sheep (Smith *et al.*, 1936; Beath *et al.*, 1939), although toxic plants in the environment may have also played roles. However, at least some of the adverse

effects on reproduction caused by excess selenium in ruminants are caused by interference with absorption and retention of copper that results in copper deficiency.

Selenosis in poultry has major effects in reproduction. Poor hatchability, embryonic deformities and embryonic death are common sequelae to selenium poisoning (Hoffman, 2002; Latshaw *et al.*, 2004; Hamilton, 2004).

## TREATMENT

The most effective treatment is to prevent excessive exposure. Although the maximal tolerable level for selenium was once set at 2 mg/kg of diet per day for all species, this has now been changed to 5 mg/kg of diet per day for ruminants (NRC, 2005). It is stated that this new tolerance for ruminants is appropriate for horses as well. Swines have a maximum tolerance level of 4 mg/kg of diet, for poultry it is set at 3 mg/kg of diet, and for fish it is set at 2 mg/kg of diet.

As there is no specific mechanism of chelation and removal of selenium in animals, the primary treatment protocol is of supportive care with both acute and chronic selenium poisoning. With chronic poisoning, it is important to understand the long-term commitment necessary to allow an animal with hoof lesions time to re-grow the hoof wall once exposure has been stopped. Especially with organic selenium's incorporation into body proteins, the time necessary to just decrease the body load of selenium, once excessive exposure has stopped, is quite long.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Selenium deficiency and toxicity are problems that veterinarians, nutritionists and animal owners need to understand. The small difference between deficiency and toxicity in terms of dietary intake makes the risk of accidental selenium poisoning high. Many areas of the world have seleniferous soils which can cause poisoning via ingestion of natural forages grown on them. As there is no specific treatment for selenium toxicosis, it is critical that appropriate education be utilized in order to minimize the risks to the livestock industry.

Future research needs to address the mechanisms of physiologic damage caused by both acute and chronic selenium poisoning with differing chemical forms of selenium. With an understanding of the cellular mechanisms of selenium poisoning, more specific means of treatment might be developed. In addition, a clearer understanding of thresholds for chronic selenium poisoning is needed.

With the knowledge that certain ruminal microbes can reduce various forms of selenium to non-absorbable forms, preventive use of these microbes in seleniferous areas to prevent systemic accumulation and poisonings should be investigated.

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## Sodium chloride (salt)

Larry J. Thompson

### INTRODUCTION

Sodium chloride is often referred to as table salt, common salt or just simply salt. Salt is an essential nutrient and can give an attractive taste to foods and feeds. It has been stated that salt is the only mineral compound for which animals can truly develop a craving.

Salt is a necessary nutrient for the health of animals and many nutrition texts divide it into separate requirements for sodium (Na) and chloride (Cl). Daily requirements for salt will increase due to lactation, exertion and increases in ambient temperatures. These same conditions will also require an increase in water intake, which must always be considered in any discussion concerning salt intake.

An excess of salt intake can lead to the condition known by various names including salt poisoning, hypernatremia, sodium ion toxicosis and water deprivation–sodium ion intoxication. The last name in this list is the most descriptive, giving both the result (sodium ion intoxication) and the most common predisposing condition (water deprivation).

### BACKGROUND

Sodium is the main cation and chloride is the main anion in the regulation of osmotic balance in the extracellular fluid (ECF) of the body. Serum sodium concentration and serum osmolality are normally maintained under precise control by homeostatic mechanisms involving thirst, antidiuretic hormone and renal reabsorption of filtered

sodium. Normal reference ranges for serum sodium in adult animals (given in mmol/l) include porcine 135–150, bovine 132–152, canine 141–152, feline 147–156 and equine 132–146 (Kaneko *et al.*, 1997). For sodium concentration measurement, mmol/l = mEq/l and can be used interchangeably. Hyponatremia is the result of the loss of excess sodium compared to loss of water and can be the result of a large number of disease processes and conditions. For a more complete explanation on the recognition and correction of hyponatremia the reader is directed to other references (Angelos and Van Metre, 1999).

Salt is normally present in animal diets at 0.5–1%. Production animals are often given free access to salt blocks or mineral mixes as supplements to the diet. Additional sources of salt may include high-saline ground water, brine or seawater. The use of whey as a feed or to produce wet mash can add dramatically to sodium intake. High concentrations of salt in the diet (up to 13%) have been used to limit feed intake of cattle. In general, animals can tolerate high concentrations of salt in the feed if they have free access to fresh water. Salt-deprived animals or animals not acclimated to high-salt feeds can overconsume these feeds making the animal prone to hypernatremia. Improperly formulated or improperly mixed feed can be an additional source of excess salt. Companion animal exposures to excess salt have included the use of salt as an emetic (this practice is no longer recommended) and the consumption of various salt-containing objects including rock salt and dough-salt mixtures. Hypernatremia has also been reported in animals treated with improperly mixed oral electrolyte solutions and remedies for diarrhea.

Excess ingested salt can be irritating to mucosal surfaces and can result in anorexia, vomiting or diarrhea.

Ingested dietary salt is approximately 90% absorbed across the gastrointestinal tract. Ingestion of excess sodium on an acute basis can result in hypernatremia with this condition being variously termed direct sodium ion toxicosis, acute sodium ion toxicosis or acute hypernatremia. Clinical signs develop within 1–2 days. The more common form of hypernatremia develops due to restricted water intake and is termed indirect sodium ion toxicosis, chronic sodium ion toxicosis or chronic hypernatremia. Clinical signs will develop over a period of 4–7 days but the early changes may be missed or ignored. There are several common reasons for restricted water intake including frozen water sources, unpalatable water sources, mechanical failure, overcrowding or naive animals and owner neglect.

## PHARMACOKINETICS AND MECHANISM OF ACTION

An increase in sodium intake leads to a rise in sodium concentration in the serum and a rapid distribution throughout the body. Osmolarity of the ECF is monitored by osmoreceptors in the hypothalamus and the body reacts to increases by stimulating thirst for increased water intake. Additionally, the release of antidiuretic hormone from the posterior pituitary will cause increased water retention by the kidneys. These responses should function to quickly restore normal osmolarity but may only be effective if the osmolar changes are gradual and sufficient water is available to the animal. As the sodium ion concentration of the serum increases, water will move out of the interstitium and intracellular fluid into the ECF along the osmotic gradient. Sodium will passively diffuse across the blood–brain barrier increasing the sodium concentration of the cerebral spinal fluid above the normal range (135–150 mmol/l). During this developing hypernatremia, the cells of the brain will also increase their intracellular osmolarity to prevent excess water loss to the ECF, which would cause cell shrinkage. If the hypernatremia develops too quickly and this protective mechanism fails, significant cell shrinkage occurs and the entire brain shrinks and pulls away from the calvarium resulting in the disruption of the blood supply to the brain. This can result in subarachnoid, subdural or intravascular hemorrhages (Hardy, 1989). In severe cellular dehydration, the result can be seizure-like activity and death. If the increase in sodium concentration of the brain cells continues, there will be an inhibition of glycolysis and a decrease in the energy available in the cell. While sodium will passively diffuse into the brain, it is an energy-requiring active process that transports sodium out. Thus the brain response to a rapid decrease in serum sodium is

delayed and the developing osmotic gradient will cause water to move into the brain causing swelling, cerebral edema and the development of clinical signs.

Changes in cellular osmolarity will occur in both acute and chronic hypernatremia situations, but changes to osmolarity on a chronic basis will involve the accumulation of more osmotically active organic compounds, termed idiogenic osmoles. These include taurine, myoinositol, glycerophosphoryl-choline, glutamate, glutamine, betaine and phosphocreatine. Maximum concentrations of idiogenic osmoles occur within 48–72 h and can account for 60% of the change in cellular osmolarity. Once the hypernatremia situation is corrected, the idiogenic osmoles will take 48–72 h to decrease back to normal levels. As in the acute situation, a rapid decrease in serum sodium will develop an osmotic gradient causing water to move into the brain with resulting cerebral edema and the development of clinical signs.

## TOXICITY

In all situations involving salt intake, the intake of water will have great impact and must also be considered. The acute toxic dose of sodium chloride is approximately 2.2 g/kg in swine, equine and bovine species with the ovine toxic dose approximately 6 g/kg (Osweiler *et al.*, 1985). Swine appear to be the most sensitive domestic animal and involve the greatest number of clinical reports. Both swine and poultry can be severely affected when water intake is severely restricted or with high-salt diets and only moderate water restriction. Increased water requirements will increase the susceptibility of lactating cows and sows to salt poisoning, making them more sensitive to sudden restrictions in water. The acute toxic dose of sodium chloride in dogs is given as 4 g/kg, but clinical signs have been reported for lesser ingestions (Barr *et al.*, 2004) and an ingestion greater than this was reported with only mild clinical signs. Horses appear to be rarely affected with classic salt poisoning but can develop it with conditions of increased salt intake and sudden water restriction. Horses are, however, subject to dehydration and electrolyte abnormalities especially under conditions of exercise and high ambient temperatures (Cohen *et al.*, 1993).

Clinical signs have best been described in swine and include loss of appetite, thirst, restlessness, pruritus and constipation. These early clinical signs can progress over several days to aimless wandering, head pressing, circling or pivoting around a limb. The animal may display seizure-like activity and assume a dog-sitting position, draw its head back in a jerking motion and fall over on its side (Osweiler *et al.*, 1985; Niles, 2004). Terminally, the



animal will be in lateral recumbency with paddling and opisthotonus. Cattle with acute excess salt intake may develop gastroenteritis, weakness, dehydration, tremors and ataxia. The cattle may appear to be blind and develop seizure-like activity or partial paralysis including knuckling over at the fetlocks. Terminally, cattle can also be in lateral recumbency with paddling and opisthotonus. Cattle can die within 24 h following the appearance of severe clinical signs. Recovered animals may drag the rear feet or knuckle over at the rear fetlock without exhibiting pain (Osweiler *et al.*, 1985). Poultry and other birds may exhibit clinical signs of depression, weakness, dyspnea and sudden death. Excess salt intake in the dog will result in vomiting within several hours of ingestion. The clinical signs can progress to diarrhea, muscle tremors and seizure-like activity. Increased severity of clinical signs in the dog have been seen when serum sodium levels have been above 180 mEq/l (Barr *et al.*, 2004).

Postmortem examination of salt-poisoned animals may include some degree of gastric irritation, including ulceration and hemorrhages. The content of the gastrointestinal tract may be abnormally dry. Histopathologic lesions may be limited to the brain and include cerebral edema and inflammation of the meninges. In swine, the appearance of eosinophilic perivascular cuffing is seen if the animal dies early in the syndrome with the lesion not found after 48 h. Brain sodium concentrations above 2000 ppm are considered diagnostic in cattle and swine. Upper normal brain sodium concentrations are 1600 ppm for cattle and 1800 ppm for swine, both on a wet weight basis. There is a paucity of data on normal brain sodium concentrations in other common domestic species but normal ranges should be similar. Serum sodium concentrations taken from the live animal will be significantly above the normal ranges listed previously. Postmortem analysis of aqueous humor, vitreous humor or cerebral spinal fluid will show a significant increase over values from normal animals (Osweiler *et al.*, 1995). Optimally, the values obtained should be compared to normal values for that species generated by the same laboratory.

## TREATMENT

Prior to the onset of clinical signs, the acute ingestion of salt can best be treated by allowing the animal full access to water and closely observing the animal for several hours. Emetics may be used in the dog if known ingestions occur and the animal is not yet showing clinical signs. However, most cases are discovered long after the excess salt ingestion or the water deprivation has occurred and the affected animals are showing obvious

clinical signs. The overriding concept of treatment is to slowly return the animal to normal water and electrolyte balance over a 2–3-day period of time. Quickly lowering the serum sodium concentration will increase the osmotic gradient between the serum and the brain with water following the gradient into the brain increasing the likelihood of severe cerebral edema. The prognosis for an animal hypernatremic from salt ingestion/water deprivation with significant clinical signs on either an acute or chronic basis should be guarded at best.

On a herd basis with large animals, water intake should be limited to 0.5% of body weight at hourly intervals until normal hydration is accomplished. Monitoring serum sodium concentration is the first step in treatment on an individual animal basis. This information can be used to correct the free water deficit (FWD) in the animal, based on the following formula:

$$FWD(1) = 0.6 \times \text{body weight (kg)} \\ \times [(\text{measured serum Na}/\text{normal serum Na}) - 1]$$

Not more than 50% of the FWD should be replaced in the first 24 h with the remaining deficit replaced in the following 24–48 h. Serum sodium levels should be lowered at a rate of 0.5–1.0 mEq/l/h, with the slower rate recommended for cases of chronic hypernatremia (Schaer, 2000). In acute hypernatremia without clinical dehydration, the use of 5% dextrose solution in combination with a loop diuretic has been suggested at 3.7 ml/kg/h to decrease serum sodium at 1 mEq/l/h (Barr *et al.*, 2004). Diuretics such as furosemide can be used to prevent the development of pulmonary edema during fluid therapy. The use of slightly hypertonic intravenous fluids has been recommended to reduce the likelihood of cerebral edema developing. Intravenous fluids should be made to approximate the serum sodium concentration of the animal, or the clinician may start with a solution containing 170 mEq/l of sodium and decrease this concentration as clinical signs improve (Angelos and Van Metre, 1999; Niles, 2004). If brain edema is suspected, the use of mannitol, dexamethasone or dimethyl sulfoxide may aid in control.

## CONCLUDING REMARKS

While the term “salt poisoning” may not be the most accurate way to describe the above syndrome, it is certainly in common usage. A similar and confusing term is “water intoxication” which has been used to describe the situation of excess water intake or infusion over a short period of time which can dramatically decrease the serum sodium concentration and make the serum hypoosmolar. Water intoxication has been used to

describe the exacerbation of cerebral edema when the correction of hypernatremia occurs too quickly. Water intoxication has also been used to describe the brain swelling and seizure-like activity which occurs when a normal animal drinks excessive amounts of water over a short period of time. In addition to the possible neurological effects, hemolysis has also been described (Middleton *et al.*, 1997). In the above situations, acute and dramatic osmotic changes are the cause.

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# Sulfur

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## INTRODUCTION

Sulfur is a necessary dietary component that can be toxic at excessive concentrations. Animal bodies are about 0.15% sulfur by weight (NRC, 1989, 2006). Sulfur is incorporated into many essential molecules, including biotin, chondroitin sulfate, cartilage mucopolysaccharides, co-enzyme A, fibrinogen, glutathione, heparin, lipoic acid, mucins and thiamine (NRC, 1989, 1998, 2006). In addition to these biologically active compounds, sulfur is an intricate component of sulfur-containing amino acids, such as methionine, cysteine, cystine, homocysteine and taurine. With the exception of thiamine and biotin, all sulfur-containing compounds in the body can be synthesized from methionine (NRC, 1996). Thus, thiamine, biotin and methionine are essential nutrients in the diet of monogastric animals, but ruminant microbes can synthesize these compounds from inorganic sulfate in the diet (Block *et al.*, 1951). Species differences are such that cats cannot synthesize taurine from methionine, making it an essential nutrient in their diets. Recommended daily dietary intakes of sulfur are 0.15%, 0.14–0.26%, 0.15–0.2% and 0.2–0.25% of the diet for horses, sheep, beef cattle and dairy cattle, respectively (NRC, 1985, 1988, 1989, 1996).

Ruminants tend to be more sensitive to the toxic effects of dietary sulfur/sulfate due to efficient microbial conversion to bioactive sulfur species in the rumen. But both dietary and water sources of sulfur/sulfate have similar toxic potential and must be factored into the total daily intake in order to establish potential risk. Dietary feed sources that can contain high sulfur concentrations, which are frequently overlooked, include distiller's byproducts, wet and dry distiller's grains and gluten feeds. And these

products can have significant batch to batch variability in sulfur content, even from the same production plant. Due to their relative inexpensive nature, compared to grains, these high sulfur materials are more and more frequently being added to ruminant diets.

In addition to dietary sulfur, other sulfur-containing compounds can be toxic. Sulfur dioxide gas from industrial waste gas, as well as hydrogen sulfide gas from manure pits, natural gas production and crude oil production, can be toxic to livestock. The toxic effects of these gaseous forms of sulfur are better summarized separately from the dietary toxicoses.

Plants can accumulate high sulfur concentrations. High sulfate water can cause a dual increase in total daily sulfur intake by way of the water and ingested proximal vegetation. Plant sulfur concentrations have been shown to increase with increasing sulfate in the soil (Reddy *et al.*, 1981; Hardt *et al.*, 1991; Leustek and Saito, 1999). In soil matrixes, sulfate can be actively reduced and precipitated; however, this only sequesters the sulfur until environmental change allows the re-oxidation of the sulfur back to sulfate. During drought conditions, precipitated sulfur in the subsurface soil is exposed to greater oxygen and potentially re-oxidized. The resultant sulfate is then bioavailable for plant uptake during subsequent periods of normal or high precipitation.

## BACKGROUND

Sulfur is a non-metal within group VIA of the periodic table. This group is sometimes referred to as the

chalcogenides or ore-formers, since many metal ores are sulfide or sulfate salts. Sulfur has an atomic number of 16, an atomic weight of 32.07 and has four different naturally occurring atomic masses from 32 to 36 (Rosman and Taylor, 1998). It can occur in four different oxidation states:  $-2$  (sulfide),  $0$  (elemental sulfur),  $+4$  (sulfite) and  $+6$  (sulfate). All valence states, except elemental sulfur, are found in biologic molecules. Sulfur is utilized in the production of sulfuric acid, fertilizers, pigments, dyes, drugs, explosives, rubber, insecticides and detergents, as well as many inorganic salts and esters. Although uniformly found in nature, industrialized countries are the largest users of sulfur materials.

## PHARMACOKINETICS/ TOXICOKINETICS

When evaluating the absorption of sulfur, the chemical form must be considered. The intestinal mucosal absorption of sulfate is via an active carrier-mediated process that is also utilized by molybdate (Mason and Cardin, 1977). Active intestinal absorption of sulfate has been shown in sheep, rats, dogs, rabbits and hamsters (Bird and Moir, 1971). Similarly, the sulfur-containing amino acids and other sulfur-containing compounds are absorbed via specific transporter mechanisms across the intestinal mucosa (NRC, 2006). These specific transport processes are specific for the individual compounds. Rumen microbes convert a percentage of dietary sulfur-containing compounds to sulfide, which can then be incorporated into microbial sulfur-containing amino acids, thiamine, biotin, other microbial sulfur metabolites, or absorbed as sulfide. In addition to gastrointestinal absorption of sulfides, hydrogen sulfide can be absorbed across respiratory epithelium. Large amounts of sulfide, as hydrogen sulfide, produced in the rumen can be eructated, inhaled and absorbed (Dougherty *et al.*, 1965). And inhaled sulfide is important in sulfur toxicosis, as sheep that had their trachea blocked to prevent eructation and inhalation of sulfide did not succumb while those without tracheal block were poisoned (NRC, 2006).

Sulfur is widely distributed in the body. All tissues in the body have significant sulfur components, with the body being made up of approximately 0.15% sulfur (NRC, 2006). Absorbed sulfides and thiomolybdates, the primary toxic sulfur metabolites, are well distributed in the body. This is evidenced by the fact that thiomolybdate can deplete tissue stores of copper and sulfides can cross the blood-brain barrier (BBB) causing neurological effects.

Sulfur-containing amino acids and sulfate are extensively metabolized in order to produce biologically utilized sulfur compounds. In comparison, absorbed sulfide is efficiently metabolized in the liver to sulfate, with a

high first pass clearance (NRC, 2006). Inhaled sulfide would not be subject to the rapid hepatic removal that occurs for that absorbed from the gastrointestinal tract, resulting in increased circulating concentration that can result in neurologic effects.

Sulfur-containing compounds are eliminated by both renal and biliary routes. Just as molybdate can compete for the intestinal absorption sites for sulfate, it can also compete for reabsorption sites in the renal tubules (Friberg and Lener, 1986). The relative quantities of sulfur elimination from renal and biliary routes can differ depending on the form ingested. In sheep, Bird (1972) found the greatest percent elimination of sulfate was via urine, while that from taurine was predominantly eliminated in the bile. Intestinally absorbed sulfide efficiently undergoes hepatic metabolism to sulfate, which is eliminated in the urine (NRC, 2006).

## MECHANISM OF ACTION

Acute oral poisoning with elemental sulfur results in formation of hydrogen sulfide, as well as many other potential metabolites. The gastric and respiratory effects are postulated to be due to the coagulative effects of rumen-produced sulfurous acids and the irritating effects of hydrogen sulfide, respectively (Julian and Harrison, 1975; Kandyliis, 1984; Gunn *et al.*, 1987). However, the exact mechanisms are not well delineated. Inhaled ruminal sulfide at high concentrations may act in a similar mechanism to high concentrations of exogenous hydrogen sulfide gas, causing acute respiratory paralysis.

The mechanism of subacute sulfur poisoning is much better researched. This condition is correlated with the reduction of the sulfate or other forms of sulfur to sulfide in the rumen (Gould *et al.*, 1991, 1997; Loneragan *et al.*, 1998). The current literature suggests that inhibition of cytochrome C oxidase, which is essential for cellular respiration, is the primary mechanism (Smith *et al.*, 1977; Beauchamp *et al.*, 1984). But cerebral vasospasms and regional ischemia could also account for the localization of the lesions (Siesjo, 1984; McAllister, 1991). Although once thought to be associated with a true thiamine deficiency from either inhibition of rumen microbial production or cleavage of thiamine (Edwin and Jackman, 1982), it has been shown that systemic thiamine concentrations are within the normal range for most animals (Olkowski *et al.*, 1992; Gould, 2000). Slight decline in the blood thiamine concentration can also be seen in some animals (Olkowski *et al.*, 1991). However, thiamine supplementation in the presence of high sulfate/sulfur-associated polioencephalomalacia (PEM) alleviates the clinical disease (Olkowski *et al.*, 1992). This would indicate that the



sulfide or some other sulfur metabolite is either competitively inhibiting the cellular uptake/utilization of thiamine or therapeutic doses of thiamine diminish the effects of sulfide on the cytochrome C oxidase enzyme.

The subacute to chronic, indirect effects of excessive sulfur are seen in ruminants, due to the efficient conversion of sulfur compounds to sulfide. The sulfide can form insoluble salts with copper and zinc (Suttle, 1974), but it can also react with molybdenum and form thiomolybdate complexes which efficiently bind copper making it non-bioavailable (Suttle, 1991). Systemic copper decreases, associated with increased sulfur/sulfate, have been reported in sheep (Moshtaghi-Nia *et al.*, 1989; Van Niekerk and Van Niekerk, 1989a, b) and cattle (Wittenberg and Boila, 1988). High forage and water sulfur have also been associated with selenium deficiency (Ivancic and Weiss, 2001). Decreased serum and wool selenium has been reported with increasing dietary sulfate (White and Somers, 1977; White, 1980). In addition, increased soil sulfate inhibits plant uptake of selenium, thereby increasing the potential for inducing a selenium deficiency in ingesting herbivores (Newman and Schreiber, 1985), which may be an important mechanism in grazing animals.

## TOXICITY

Toxicity of sulfur can be divided into three main categories that are likely to be encountered. The first is acute oral poisoning. The second is subacute to chronic direct toxicosis. And the third is subacute to chronic indirect toxicosis, as a secondary interference with other essential minerals that result in deficiencies.

Reports of acute oral sulfur poisoning are scarce in the literature. In a group of Holstein heifers, sulfur ingested at 0.85–3.8 g/kg body weight resulted in high morbidity and moderate mortality (Gunn *et al.*, 1987), while 20 heifers given 250 g sulfur in grain had high mortality (Julian and Harrison, 1975). Ewes fed a barley–sulfur mix that provided approximately 40–45 g sulfur/ewe were poisoned (White, 1964). Five horses administered 300 g sulfur succumbed to sulfur poisoning (Ales, 1907).

Clinical and pathologic manifestations of acute oral sulfur poisoning are similar across species (White, 1964; Julian and Harrison, 1975; Gunn *et al.*, 1987). Abdominal pain, colic, rumen stasis, fetid diarrhea, dehydration, metabolic acidosis, tachypnea, recumbency and hydrogen sulfide smell are expected clinical signs. Irritation, edema and hemorrhage of the gastrointestinal tract and respiratory tract also should be expected. In addition, renal tubular necrosis can be seen.

Monogastric animals are much less susceptible to the subacute direct and indirect toxic effects of excessive

sulfur intake than ruminants. Pigs can tolerate 1000 mg/l sulfur in the drinking water with only a mild cathartic effect (Paterson *et al.*, 1979) and 0.42% in the diet for several months without adverse effects (Dale *et al.*, 1973). Similarly, chicks had decreased growth rates at 1.2% dietary sulfur (Leach *et al.*, 1960). And chickens had decreased egg production, decreased feed intake and deaths at 4000 mg/l sulfate in their drinking water (Adams *et al.*, 1975). Since the indirect toxic effects of excessive sulfur are related to rumen conversion to sulfide, these effects are not observed in monogastric animals.

In contrast, for sheep, beef cattle and dairy cattle the maximum tolerable content of sulfur in the total diet is 0.4% (NRC, 1985, 1988, 1996), while concentrations slightly below this tolerable content can occasionally result in toxic effects (Gould *et al.*, 1991). Since dietary toxicity is not mutually exclusive, as sulfur and sulfate ion can have the same clinical effects, total doses of sulfur/sulfate from both water and dietary material must be taken into account when evaluating potential toxicity (Suttle, 1974). For example, water sulfate content of 500 mg/l would provide approximately half of the recommended maximal daily intake for ruminants. In ruminants, the typical clinical presentation of sulfur poisoning is one of ataxia, weakness, blindness, recumbency, seizures and death.

Subacute ingestion of toxic doses of sulfate/sulfur has been associated with PEM, a necrotizing lesion of the brain (Beke and Hironaka, 1991; Gould *et al.*, 1991; Olkowski *et al.*, 1992; Hamlen *et al.*, 1993; McAllister *et al.*, 1997). Gross and histologic lesions are primarily in the brain, but ruminal changes can be observed. Gross pathologic lesions include a darkening of the rumen contents from precipitated sulfide salts, swelling of the cerebral hemispheres, softening of the cerebral hemispheres and yellow discoloration of the cortical gray matter. Histological lesions include necrosis of the cortical gray matter and occasional areas of necrosis in the thalamus or midbrain. The clinical condition can be an additive effect of the total sulfur in the diet and sulfate in the drinking water (Beke and Hironaka, 1991). PEM has been reported to be associated with high sulfur/sulfate ingestion in cattle (Beke and Hironaka, 1991; Gould *et al.*, 1991; Hamlen *et al.*, 1993), pigs (Dow *et al.*, 1963) and sheep (Olkowski *et al.*, 1992). However, sodium ion poisoning in pigs was likely the primary causative factor, as the exposure was to sodium sulfate.

The peak rumen production of hydrogen sulfide can be somewhat delayed from the time of initiating high sulfur intake. Peak rumen gas cap sulfide occurred at 1–3 weeks after placing cattle on a high sulfur diet (Gould *et al.*, 1997). But, continuing exposure resulted in a gradual decline in the gas cap sulfide content. This would indicate an adaptation of the rumen microbes to favor direct utilization of the sulfide or diminished rates of production.

Subacute to chronic sulfur-induced mineral deficiencies can result in severe health problems. Copper

deficiency can cause poor growth, weakness, poor immune function, poor reproductive function and death. In addition, sulfur-induced copper deficiency may play a role in PEM (Gooneratne *et al.*, 1989). Severe copper deficiency also causes myelin degeneration (enzootic ataxia) in lambs, deer and other ruminants (Cordy, 1971; Faye *et al.*, 1991; Audige *et al.*, 1995). Sulfate-induced selenium deficiency can cause poor growth, weakness, poor immune function, poor reproductive function, damage to the cardiac or skeletal muscles and death.

## TREATMENT

Treatment for acute sulfur poisoning is predominantly supportive in nature, with removal of the causative material, as well as administration of fluids and electrolytes. However, the human literature points to successful treatment of acute hydrogen sulfide poisoning by induction of methemoglobinemia with nitrite to allow for the formation of sulfmethemoglobin, similar to therapeutic protocols for treatment for cyanide poisoning (Stine *et al.*, 1976; Peters, 1981). This type of therapy may also be beneficial in the treatment of subacute direct sulfur poisoning. Since sulfides act on and split disulfide bridges, the use of oxidized glutathione or other simple disulfide compounds could be protective or antidotal (Smith and Abbanat, 1966). The use of thiamine in the treatment of PEM has been suggested to be beneficial, even though there is no overt thiamine deficiency. The primary treatment of indirect mineral deficiencies resultant from high sulfur intake would also include enhanced supplementation of copper and potentially selenium. In some cases in which limitation of water or forage sulfur intake is not possible, use of chelated copper and selenium becomes important in order to bypass the binding of copper in the rumen and competitive inhibition of selenate from the intestinal tract.

In addition to direct treatment, management can play a role in the prevention of sulfur poisoning. Testing of water and dietary materials will identify high sulfur prior to utilization. With this information, management strategies can be utilized that would incorporate the sulfur at gradually increasing content in an attempt to allow for microbial adaptation.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Although much is known about the different clinical disease conditions that excessive sulfur can cause, treatment remains only somewhat effective. Thus, it is important

to evaluate the use of nitrite- or disulfide-containing compounds in the treatment of domestic animals.

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## Zinc

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## INTRODUCTION

Zinc (Zn) is a transitional metal in group XII on the periodic chart and is the fourth most commonly used metal today. It is a moderately reactive metal with a common valence state of +2. It is also an essential element in mammals and birds and is a component of approximately 200 metalloenzymes. Although zinc has an essential role in nutrition and consequences of nutritional deficiency, this chapter will focus on its toxicity.

## BACKGROUND

As the fourth most commonly used metal it is expected to be found in a variety of places with a multitude of uses. Zinc is an economical metal to use and is relatively non-toxic. Table 44.1 details some of those uses. Since zinc has nutritional qualities, it is not uncommon to find it added as a supplement to feed. Problems with toxicity generally occur when the levels are 1000 parts per million (ppm) or greater.

PHARMACOKINETICS/  
TOXICOKINETICS

Ingested zinc is primarily absorbed from the duodenum and the intestine by a carrier-mediated mechanism. Approximately 25 to 50% of ingested zinc is absorbed.

TABLE 44.1 Uses and purposes of zinc

Zinc is used in:	Its purpose is:
Galvanized steel	Prevents corrosion
Parkerize steel	Prevents rust and corrosion
Used in numerous metal alloys	Brass, nickelled silver, typewriter metal, various soldering formulas, German silver
Primary metal	American pennies, nuts and bolts
Die casting	Automotive industry
Zinc oxide	Paints, sun protectants, rubber activator, diaper rash ointments
Wall tiles	Germicidal properties
Zinc chloride	Wood preservative and deodorant
Zinc methyl ( $\text{Zn}(\text{CH}_3)_2$ )	Number of organic syntheses
Zinc stearate	A lubricative plastic additive

However, absorption of zinc is influenced by many factors, including whether or not food is in the stomach. Plant phytates can bind zinc and in an alkaline pH environment can form insoluble complexes. Similarly zinc absorption is decreased in the presence of phosphates and calcium in the diet. However, the presence of some peptides, amino acids and ethylenediamine tetra acetic acid disodium (EDTA) may cause an increase in absorption. Generally, the stomach acid provides for rapid release of zinc from ingested metallic objects.

Once zinc has been absorbed it is bound to plasma albumin and to macroglobulins, and transported to the liver. Zinc is extracted by the liver and returned to the bloodstream for distribution to the liver, pancreas, kidney and spleen which all rapidly accumulate the zinc. These tissues, as well as muscle and prostate, are induced to synthesize metallothionein in the cells.



Under normal dietary conditions, excess zinc is excreted through the feces. Nevertheless, excretion of zinc, especially in toxic situations, is limited. Excretion occurs through the bile and feces but may also occur through urine (Abdel-Mageed and Oehme, 1990) and saliva.

## MECHANISM OF ACTION

The mechanism of action producing clinical signs is not well defined or understood. The characteristic clinical signs are more easily recognized. The most recognized abnormality observed is severe intravascular hemolysis and gastroenteritis. This may be a result of excess zinc interfering with copper and iron storage and utilization, resulting in a suppression of hematopoiesis.

Diets high in zinc interfere with hepatic copper storage and may compete with calcium for intestinal absorption.

## TOXICITY

The different forms of zinc have different toxicities. The zinc salts have a median lethal dose (LD<sub>50</sub>) of approximately 100 mg/kg body weight. Zinc oxides are less toxic. Zinc oxides are frequently found in ointments, such as for preventing sunburn or treating diaper rash. Dogs often ingest it when it is applied topically to them or to someone they can lick it off. Dogs also ingest the ointment by chewing on the tube container. It has been estimated that the toxic dose is approximately 108 grams of zinc in the dog (Breitschwerdt *et al.*, 1986).

The ingestion of pennies, which are 96% zinc (Latimer *et al.*, 1989), by dogs has resulted in the development of a subacute zinc toxicosis. The pennies remain in the acidic environment of the stomach and slowly release metallic zinc. It is unclear how many pennies are required to produce a toxicosis. The pennies can be vomited or passed in the feces prior to presentation at the veterinary clinic.

Other forms of zinc-containing hardware such as transport kennel bolts or machine nuts and bolts have caused problems in animals. Analysis of these objects has shown them to be about 97% zinc (Breitschwerdt *et al.*, 1986; Torrance and Fulton, 1987). They remain in the stomach producing a subclinical toxicosis or are vomited or passed out prior to the animal's presentation.

Other sources of toxicity have been galvanized wire used in cages in aviaries (Reece *et al.*, 1986) and for housing ferrets (Straube *et al.*, 1980). The toys an animal selects may be made almost entirely of zinc (Bexfield *et al.*, 2007), and may not be a toy necessarily designed for animals.

An additional component of zinc toxicosis is acute renal failure observed in some dogs (Breitschwerdt *et al.*, 1986). The clinical findings include hypercreatinemia, azotemia, hyperphosphatemia and granular casts in the urine (Breitschwerdt *et al.*, 1986; Torrance and Fulton, 1987; Latimer *et al.*, 1989). Other signs of acute intoxication are pancreatitis and acute arthritis. Also, non-viable newborns may be observed with zinc intoxication.

Foals are susceptible to zinc intoxication. It appears to be a chronic zinc toxicosis. Initially, the foals have non-painful joint enlargement lasting 7–21 days. They are reluctant to rise and have stiff gait and increased joint fluid (Gunson *et al.*, 1982).

Other livestock have early signs of lethargy and anorexia followed by diarrhea. Later, they display a decreased rate of gain or decreased milk production. As the toxic condition progresses, the animals have anemia and icterus. Other signs include exophthalmia, polydipsia, polyphagia and seizures.

Generally most species display some degree of hemolytic anemia, often with an erythrocytic regenerative response. Additionally, there is renal damage with hematuria, urinary casts and proteinuria. Diagnostically, there may be a radiodense area in the gastrointestinal tract indicating the presence of zinc or some other metal such as lead. Postmortem lesions include renal tubular necrosis, hepatocyte necrosis and gastroenteritis.

## TREATMENT

As with many metal intoxications, removal of the source of the toxin from the animal, followed by supportive therapy, are critical to the patient's recovery. This procedure with zinc will result in dramatic drops in serum and tissue levels within a relatively short time. Removal of the source will allow the normal excretory pathways to work appropriately.

Particularly when treating foals it is important to evaluate the copper status of the animal. Copper and zinc seem to work in tandem and adding copper to copper-deficient animals may help decrease the overriding effects of zinc.

Supportive care is critically important as the gastroenteritis and anemia must be addressed. The vomiting, in species capable of vomiting, has usually resulted in severe gastroenteritis. Vomiting may be controlled with metoclopramide (0.2–0.4 mg/kg) administered every 6 hours intramuscularly, subcutaneously or *per os*. Blood transfusions may be in order to address the anemia. Additionally, fluid therapy with a balanced solution such as lactated Ringer's solution should be considered. Good supportive care includes continuous monitoring of

various blood parameters such as red blood cells, platelets, packed cell volume and serum chemistries including liver enzymes, serum urea nitrogen and electrolytes. Treatment efforts must be directed at correcting pancreatic, renal and/or liver dysfunctions that are so closely associated with zinc intoxication.

Chelation therapy is an option in the patient's care. Chelation therapy is effective but not without risk and so must be evaluated with regard to the patient's condition. Patient conditions requiring evaluation include the hydration status, the degree of dysfunction of the excretory organs and the severity of the serum zinc concentration. Another important factor is whether the offending object could be removed from the patient. The most commonly suggested chelator is calcium disodium EDTA. Calcium disodium EDTA is most commonly used to treat lead intoxication and the dose for zinc intoxication has thus been extrapolated (100mg/kg, divided into four doses per day administered subcutaneously, diluted in 5% dextrose in water to decrease local irritation). Especially with chelation therapy, daily monitoring of the patient is essential to determine the length of therapy.

## CONCLUSIONS

Evidence of pancreatitis and liver and kidney dysfunction must be considered along with gastroenteritis and a hemolytic event when considering the differentials. The clinical sign of acute gastroenteritis is common to viral and bacterial diseases, parasitic diseases and various neoplasms. Gastroenteritis and a hemolytic event must be differentiated from other metal intoxications such as

copper poisoning. Additional considerations include acetaminophen or onion intoxication, mustard poisoning, immune-mediated diseases and certain snake bites.

As zinc is a very economically useful metal it is likely to be found in more products that animals are exposed to in various fashions. It would be beneficial to have a larger array of safe chelation products. Unfortunately, the importance of chelation therapy outweighs the available markets for the product. Therefore it is unlikely research dollars will be directed at finding new chelation therapies.

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# Organophosphates and carbamates

Ramesh C. Gupta and Dejan Milatovic

## INTRODUCTION

Organophosphates (OPs) and carbamates (CMs) are commonly used as pesticides in agriculture, industry and around the home/garden throughout the world. In addition, these chemicals are used as parasiticides in veterinary medicine. Both types of chemicals produce their toxicity by virtue of inhibition of acetylcholinesterase (AChE) enzyme, which terminates the action of the neurotransmitter acetylcholine (ACh) at the synapses in nervous tissue and at the neuromuscular junctions (NMJs). These chemicals are referred to as “anticholinesterases.” Some of the OPs with strong AChE inhibiting potential are also used as nerve agents or nerve gases in chemical warfare.

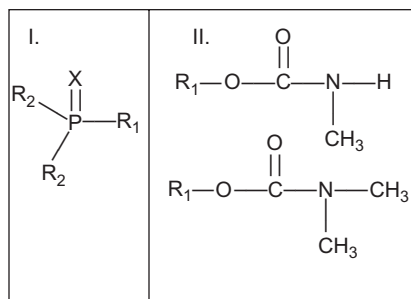
Since many compounds of both classes are extremely toxic and lack species selectivity, their inadvertent/accidental use continues to pose a threat to the environment, human and animal health, wildlife and aquatic systems. Small animals often encounter poisoning with these insecticides by malicious activity, while livestock by ingesting freshly sprayed crop or contaminated feed. Although these compounds are neurotoxicants, they produce a variety of cholinergic and non-cholinergic effects. The latest evidence suggests that while cholinergic mechanisms play a critical role in the initial stage of toxicity, neuronal damage/death appears to occur through non-cholinergic mechanisms. OPs and CMs are discussed here together because they produce similar toxic effects in poisoned animals. This chapter covers various aspects of toxicity of OP and CM compounds and therapeutic measures in animals. For more details, readers are referred to some recent

publications elsewhere (Gupta, 2006, 2009; Satoh and Gupta, 2010; Gupta and Milatovic, 2011).

## BACKGROUND

The first OP compound, tetraethyl pyrophosphate, was synthesized in 1854 by Philipe de Clermont. In 1932, Lange and Kruger described the synthesis of dimethyl and diethyl phosphorofluoridate. Based on the chemistry of these compounds, Gerhard Schrader (a chemist at the I.G. Farbenindustrie) led the exploration of OP class of compounds that could be used as insecticides. One of the earliest OP insecticides synthesized by Schrader was parathion, which is still used worldwide. Prior to World War II (WWII), the German Ministry of Defense developed highly toxic OP compounds of G series (tabun, sarin and soman) and diisopropyl phosphorofluoridate. In the 1950s, OP compounds with super toxicity of V series, such as VX and VR, were synthesized in the UK and the Soviet Union. After WWII, thousands of OPs have been synthesized in the search for compounds with species selectivity, i.e., more toxicity to insects and less toxicity to mammals. Malathion is an example. This compound has been used for more than half a century as the most popular insecticide. Today, more than 200 OPs are in use for a variety of purposes, such as protection of crops, grains, gardens, homes and public health.

The first carbamate compound, physostigmine (eserine alkaloid), was isolated from calabar beans (ordeal poison) of a perennial plant *Physostigma venenosum* in the



**FIGURE 45.1** General structure for organophosphorus (I) and carbamate (II) insecticides. (Adapted from [Timchalk, 2006](#).)

mid-1860s. The compound was used to treat glaucoma. About 50 years later, an aromatic ester of carbamic acid, neostigmine, was synthesized and used in the treatment of myasthenia gravis. Most of the carbamates (esters of carbamic acid) that are used as pesticides were synthesized in the 1960s and 1970s. Carbaryl was the first CM compound

used as an insecticide. The most toxic compound of this class, aldicarb, was synthesized by mimicking the structure of ACh. Like OPs, thousands of CMs have been synthesized, but less than two dozen compounds have been used practically. Today, CMs are preferred for pesticide use over OPs because some OPs have been found to be extremely toxic, while others cause delayed neuropathy in animals as well as in humans. In essence, both OPs and CMs have broad applications in agriculture and veterinary medicine and as a result of their indiscriminate use acute poisonings often result in animals, birds, fish and wildlife.

Basic structures of organophosphorus and carbamate compounds are shown in [Figure 45.1](#). There are at least 13 types of OPs (see [Table 45.1](#)). Despite differences in chemical structures, all OPs share one thing in common: they all have a pentavalent phosphorus atom and a characteristic phosphoryl bond ( $P=O$ ) or thiophosphoryl bond ( $P=S$ ). Essentially, OPs are esters of phosphoric acid with varying combinations of oxygen, carbon, sulfur and/or nitrogen attached. Of course, the chemistry of these compounds

**TABLE 45.1** Types of organophosphates

Type of OP	Chemical structure	Example
Phosphates	$\begin{array}{c} \text{O} \\    \\ \text{RO}-\text{P}-\text{OR} \\   \\ \text{OR} \end{array}$	Chlorfenvinphos Dichlorvos Monocrotophos
Phosphonates	$\begin{array}{c} \text{O} \\    \\ \text{RO}-\text{P}-\text{R} \\   \\ \text{OR} \end{array}$	Trichlorfon
Phosphinates	$\begin{array}{c} \text{O} \\    \\ \text{R}-\text{P}-\text{R} \\   \\ \text{OR} \end{array}$	Gluphosinate
Phosphorothioates ( $S=$ )	$\begin{array}{c} \text{S} \\    \\ \text{RO}-\text{P}-\text{OR} \\   \\ \text{OR} \end{array}$	Bromophos Diazinon Fenthion Parathion Pirimiphos-methyl
Phosphonothioates ( $S=$ )	$\begin{array}{c} \text{S} \\    \\ \text{RO}-\text{P}-\text{R} \\   \\ \text{OR} \end{array}$	EPN Leptophos
Phosphorothioates ( $S$ -substituted)	$\begin{array}{c} \text{O} \\    \\ \text{RS}-\text{P}-\text{OR} \\   \\ \text{OR} \end{array}$	Demeton-S-methyl Echothiophate

(Continued)



TABLE 45.1 (Continued)

Type of OP	Chemical structure	Example
Phosphonothioates (S-substituted)	$\begin{array}{c} \text{O} \\    \\ \text{RS}-\text{P}-\text{R} \\   \\ \text{OR} \end{array}$	VX
Phosphorodithioates	$\begin{array}{c} \text{O} \\    \\ \text{RS}-\text{P}-\text{SR} \\   \\ \text{OR} \end{array} \quad \text{or} \quad \begin{array}{c} \text{S} \\    \\ \text{RS}-\text{P}-\text{OR} \\   \\ \text{OR} \end{array}$	Azinphos-ethyl Azinphos-methyl Dimethoate Disulfoton Malathion Methidathion
Phosphorotrithioates	$\begin{array}{c} \text{O} \\    \\ \text{RS}-\text{P}-\text{SR} \\   \\ \text{SR} \end{array}$	DEF (tribufos)
Phosphoramidates	$\begin{array}{c} \text{O} \\    \\ \text{RO}-\text{P}-\text{N} \begin{array}{l} \nearrow \text{R} \\ \searrow \text{R} \end{array} \\   \\ \text{OR} \end{array}$	Fenamiphos
Phosphoramidothioates	$\begin{array}{c} \text{S} \\    \\ \text{RO}-\text{P}-\text{N} \begin{array}{l} \nearrow \text{R} \\ \searrow \text{R} \end{array} \\   \\ \text{OR} \end{array} \quad \text{or} \quad \begin{array}{c} \text{O} \\    \\ \text{RS}-\text{P}-\text{N} \begin{array}{l} \nearrow \text{R} \\ \searrow \text{R} \end{array} \\   \\ \text{OR} \end{array}$	Methamidophos Isofenphos
Phosphorofluoridates	$\begin{array}{c} \text{O} \\    \\ \text{RO}-\text{P}-\text{F} \\   \\ \text{OR} \end{array}$	Diisopropyl phosphorofluoridate (DFP)
Phosphonofluoridates	$\begin{array}{c} \text{O} \\    \\ \text{RO}-\text{P}-\text{F} \\   \\ \text{R} \end{array}$	Cyclosarin Sarin Soman

Note: Adapted from Marrs (1993).

is much more complex. The OPs that are derivatives of phosphoric or phosphonic acid possess anticholinesterase activity, unlike those that are derivatives of phosphinic acid. Usually, OP compounds have two alkyl substituents and an additional substituents group (leaving group, which is more labile to hydrolysis than the alkyl group). Basically, some OPs (such as dichlorvos, monocrotophos and trichlorfon) are direct AChE inhibitors, while those of

phosphorothioates type (such as bromophos, diazinon, fenitrothion and parathion) possess minimal or no anticholinesterase (anti-AChE) activity and require desulfuration to the analogous oxon before acquiring anti-AChE activity and hypercholinergic effects. Also, OPs which are used as defoliant (S,S,S-tributyl phosphorotrithioate and S,S,S-tributyl phosphorotrithioite) and herbicides (glyphosate and gluphosinate) are of very low mammalian toxicity.

TABLE 45.2 A brief chemical description of commonly used OP pesticides

Chemical	Chemical name	Mol. wt.	Oral LD <sub>50</sub> in rat (mg/kg)	Dermal LD <sub>50</sub> in rabbit (mg/kg)
Acephate	<i>O,S</i> -dimethyl acetylamiidothiophosphate	183.17	866	>2000
Azinphos-ethyl	<i>O,O</i> -diethyl <i>S</i> -[(4-oxo-1,2,3-benzotriazin-3(4 <i>H</i> )-yl)methyl] dithiophosphate	345.38	13	250
Azinphos-methyl	<i>O,O</i> -dimethyl <i>S</i> -[(4-oxo-1,2,3-benzotriazin-3(4 <i>H</i> )-yl)methyl] dithiophosphate	317.32	5	220
Bromophos	<i>O</i> -(4-bromo-2,5-dichlorophenyl) <i>O,O</i> -dimethyl thiophosphate	366.00	1600	2188
Cadusaphos	<i>S,S</i> -di- <i>sec</i> -butyl <i>O</i> -ethyl dithiophosphate	270.40	391	143
Carbophenothion	<i>S</i> -[(4-chlorophenyl)thio] methyl] <i>O,O</i> -diethyl dithiophosphate	342.87	6	22
Chlorethoxyphos	<i>O,O</i> -diethyl <i>O</i> -(1,2,2,2-tetrachloroethyl) thiophosphate	336.00	1.8	12.5
Chlorfenvinphos	2-chloro-1-(2,4-dichloro-phenyl)vinyl diethyl phosphate	359.57	12	3200
Chlorpyrifos	<i>O,O</i> -diethyl <i>O</i> -(3,5,6-trichloropyridin-2-yl) thiophosphate	350.59	135	2000
Chlorpyrifos-methyl	<i>O,O</i> -dimethyl <i>O</i> -(3,5,6-trichloropyridin-2-yl) thiophosphate	322.53	941	2000
Coumaphos	<i>O</i> -(3-chloro-4-methyl-2-oxo-2 <i>H</i> -chromen-7-yl) <i>O,O</i> -diethyl thiophosphate	362.77	13	–
Crotoxyphos	1-phenylethyl (2 <i>E</i> )-3-[(dimethoxyphos-phoryl)oxy]but-2-enoate	314.27	125	385
Cyanophos	<i>O</i> -(4-cyanophenyl) <i>O,O</i> -dimethyl thiophosphate	243.22	610	800
Demeton-O	<i>O,O</i> -diethyl <i>O</i> -[2-(ethylthio)ethyl] thiophosphate	258.34	2.5	8
Diazinon	<i>O,O</i> -diethyl <i>O</i> -(2-isopropyl-6-methylpyrimidin-4-yl) thiophosphate	304.35	300	379
Dichlorvos	2,2-dichlorovinyl dimethyl phosphate	220.98	25	59
Dicrotophos	(1 <i>E</i> )-3-(dimethylamino)-1-methyl-3-oxoprop-1-en-1-yl dimethyl phosphate	237.19	22	223
Dimethoate	<i>O,O</i> -dimethyl <i>S</i> -[2-(methylamino)-2-oxoethyl] dithiophosphate	229.26	250	400
Disulfoton	<i>O,O</i> -diethyl <i>S</i> -[2-(ethylthio)ethyl] dithiophosphate	274.40	2	6
Ethion	<i>O,O,O',O'</i> -tetraethyl <i>S,S'</i> -methylene bis(dithiophosph-ate)	384.48	27	915
Famphur	<i>O</i> -[4-[(dimethylamino)sulfonyl]phenyl] <i>O,O</i> -dimethyl thiophosphate	325.34	35	2730
Fenamiphos	ethyl 3-methyl-4-(methylthio)phenyl isopropylamidophosphate	303.36	15.3	–
Fenitrothion	<i>O,O</i> -dimethyl <i>O</i> -(3-methyl-4-nitrophenyl) thiophosphate	277.23	250	1300
Fenthion	<i>O,O</i> -dimethyl <i>O</i> -[3-methyl-4-(methylthio)phenyl] thiophosphate	278.33	255	330
Fonofos	<i>O</i> -ethyl <i>S</i> -phenyl ethylphosphonodi-thioate	246.33	8	25
Glyphosate	<i>N</i> -(phosphonomethyl)-glycine	169.07	4300	>5000
Gluphosinate ammonium	2-amino-4-[hydroxy(methyl)phosphoryl]butanoic acid ammoniate	198.16	2000	>4000
Glyphosine	<i>N,N</i> -bis(phosphonomethyl)glycine	263.08	3925	>5010
Isazophos	<i>O</i> -(5-chloro-1-isopropyl-1 <i>H</i> -1,2,4-triazol-3-yl) <i>O,O</i> -diethyl thiophosphate	313.75	40	>3100
Isufenphos	isopropyl 2-[[ethoxy(isopropylamino)phosphorothioyl]oxy]benzoate	345.40	32	162
Malathion	diethyl 2-[(dimethoxyphos-phorothioyl)thio] succinate	330.36	885	4000
Methamidophos	<i>O,S</i> -dimethyl amidothiophosphate	141.13	13	110
Methodathion	<i>S</i> -[(5-methoxy-2-oxo-1,3,4-thiadiazol-3(2 <i>H</i> )-yl)methyl] <i>O,O</i> -dimethyl dithiophosphate	302.33	25	200
Methyl parathion	<i>O,O</i> -dimethyl <i>O</i> -(4-nitrophenyl) thiophosphate	263.21	9	63
Mevinphos	methyl (2 <i>E</i> )-3-[(dimethoxyphos-phoryl)oxy]but-2-enoate	224.15	3	16
Monocrotophos	dimethyl (1 <i>E</i> )-1-methyl-3-(methylamino)-3-oxoprop-1-en-1-yl phosphate	223.16	8	354
Omethoate	<i>O,O</i> -dimethyl <i>S</i> -[2-(methylamino)-2-oxoethyl] thiophosphate	213.19	50	1400
Paraoxon	diethyl 4-nitrophenyl phosphate	275.19	1.8	–
Parathion	<i>O,O</i> -diethyl <i>O</i> -(4-nitrophenyl) thiophosphate	291.26	3	6.8
Phenthoate	ethyl [(dimeth-oxyphosphoro-thioyl)thio] (phenyl)acetate	320.36	200	4000
Phorate	<i>O,O</i> -diethyl <i>S</i> -[(ethylthio)methyl] dithiophosphate	260.38	1.6	2.5
Phosmet	<i>S</i> -[(1,3-dioxo-1,3-dihydro-2 <i>H</i> -iso-indol-2-yl)methyl] <i>O,O</i> -dimethyl dithiophosphate	317.32	147	3160
Phosphamidon	(1 <i>Z</i> )-2-chloro-3-(diethylamino)-1-methyl-3-oxoprop-1-en-1-yl dimethyl phosphate	299.69	15	125
Phoxim	phenylglyoxalo-nitrile oxime, <i>O,O</i> -diethyl phosphorthioate	289.30	1845	1126
Profenofos	<i>O</i> -(4-bromo-2-chlorophenyl) <i>O</i> -ethyl <i>S</i> -propyl thiophosphate	373.63	400	472
Propetamphos	isopropyl (2 <i>E</i> )-3-[(ethylamino)(methoxy)phosphorothioyl]oxy} but-2-enoate	281.31	82	2300
Quinalphos	<i>O,O</i> -diethyl <i>O</i> -quinoxalin-2-yl thiophosphate	298.30	65	340
Ronnel	<i>O,O</i> -dimethyl <i>O</i> -(2,4,5-trichlorophenyl) thiophosphate	321.55	1250	2000
Sulfotepp	<i>O,O,O,O</i> -tetraethyl dithiodiphosphate	322.32	5	–
Sulprofos	<i>O</i> -ethyl <i>O</i> -[4-(methylthio)phenyl] <i>S</i> -propyl dithiophosphate	322.45	107	820
Terbufos	<i>S</i> -[( <i>tert</i> -butylthio)methyl] <i>O,O</i> -diethyl dithiophosphate	288.43	1.6	1
Triazophos	<i>O,O</i> -diethyl <i>O</i> -(1-phenyl-1 <i>H</i> -1,2,4-triazol-3-yl) thiophosphate	313.32	83	280
Trichlorfon	dimethyl (2,2,2-trichloro-1-hydroxyethyl)phosphonate	257.44	630	>2100

## OP PESTICIDES

The majority of OP compounds are used as pesticides, and a brief chemical description for commonly used compounds is given in [Table 45.2](#).

Chemical structures of some of the commonly used OP pesticides are shown in [Figure 45.2](#).

## OP NERVE AGENTS/GASES

OP nerve agents include tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), venom toxin (VX) and Russian VX (VR). These compounds are highly toxic and pose continuous threats for the lives of humans as well as animals, because they can be used as chemical weapons of mass destruction (WMD). So far these agents have been used by dictators and terrorists. In some incidents, animals have been victims of military operations. These compounds produce toxicity by directly inhibiting AChE, and are much more potent than OP pesticides, as they cause lethality to animals in the micrograms range. Their chemical structures are shown in [Figure 45.3](#). For details of toxicity of these compounds, refer to [Watson et al. \(2006, 2009\)](#) and [Radilov et al. \(2009\)](#).

## CARBAMATES

The carbamate (CM) compounds are esters of carbamic acid. Unlike OPs, CM compounds are not structurally complex. Chemical structures of some commonly used CM insecticides are shown in [Figure 45.4](#), and a brief toxicological data of CMs is provided in [Table 45.3](#). For the details of CMs, readers are referred to [Gupta \(2006\)](#) and [Gupta and Milatovic \(2011\)](#). Currently, the volume of CMs used exceeds OPs because they are relatively safer than OPs.

## PHARMACOKINETICS OF OPs AND CMs

Pharmacokinetics deals with the rate limiting processes of absorption, distribution, metabolism and excretion (ADME). The ADME of OP and CM insecticides in animals have been described ([Tomokuni et al., 1985](#); [Gupta, 1994](#); [Wu et al., 1996](#); [Timchalk, 2006, 2010](#); [Gupta et al., 2011](#)). These insecticides gain entry into the body mainly through oral, dermal or inhalation exposure. Ingestion encounters with contaminated feed/food with pesticides

residue, while dermal exposure is more relevant when these insecticides are used as ectoparasiticides in the form of dust, dip or oily solution. Inhalation of airborne insecticides occurs during or soon after aerial spray, particularly due to chemical drift. Once the insecticide reaches at a portal of entry, it is available for absorption. It is established that following absorption, these insecticides are well distributed in tissues throughout the body. Being lipophilic, maximum levels of these compounds are usually found in the adipose tissue and brain.

In terms of metabolism, OP insecticides may follow either activation or detoxification, or both. Activation implies that the metabolite is more toxic than the parent compound, e.g., the conversion of malathion to malaoxon. This process is often referred to as "lethal synthesis." On the other hand, detoxification implies that the metabolite is less toxic than the parent compound, e.g., the conversion of malathion to malathion monoacid and malathion diacid. Unlike OPs, CMs are metabolized to less toxic or nontoxic metabolites. However, some of the metabolites of carbamates are quite toxic. For example, the two major metabolites of carbofuran (3-hydroxycarbofuran and 3-ketocarbofuran) have a significant impact on overall toxicity of carbofuran ([Gupta, 1994](#)).

A bulk of the metabolic activation and detoxification of OPs and CMs occur within the liver ([Tang et al., 2005](#); [Sogorb and Vilanova, 2010](#); [Vacondio et al., 2010](#)). Due to extensive metabolism of these insecticides in the body, only few metabolites are excreted in the urine that can be used as biomarkers of insecticides exposure. Residues of some OPs and CMs can also be detected in the feces, saliva and milk. In the case of dead animals, residue of OPs and CMs is likely to be detected in the GI content (following ingestion), adipose tissue and brain.

## MECHANISM OF ACTION

OP and CM insecticides share a common mode of toxicological action associated with their ability to inhibit the AChE enzyme within the nervous tissue and at the neuromuscular junction (NMJs). Both types of insecticides have a high affinity for binding to and inhibiting AChE, an enzyme specifically responsible for the hydrolysis of the neurotransmitter ACh. Since the cholinergic system is widely distributed within both the central and peripheral nervous systems, chemicals that inhibit AChE are known to produce a broad range of well-characterized symptoms of anticholinesterases. A graphic representation for the comparison of the AChE inhibition dynamics for the interaction of ACh, carbaryl (CM) or chlorpyrifos-oxon (OP) with AChE is shown in [Figure 45.5](#) ([Timchalk, 2006](#)). The cholinesterases (ChE) are serine hydrolases that catalyze the breakdown of ACh through an

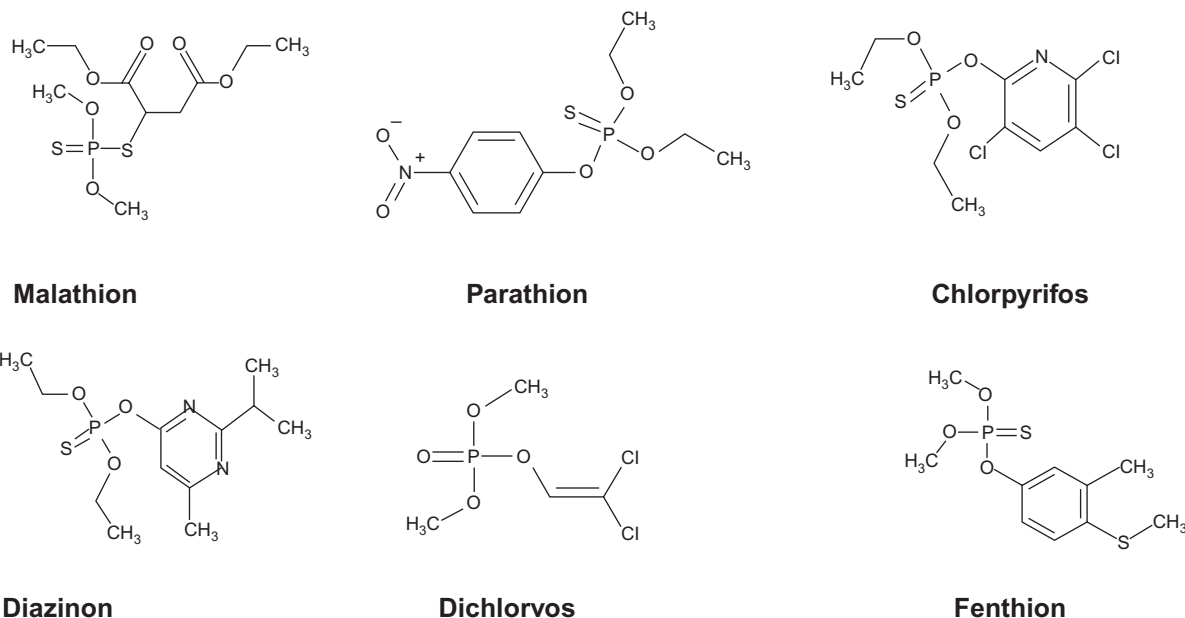


FIGURE 45.2 Chemical structures of commonly used OP pesticides.

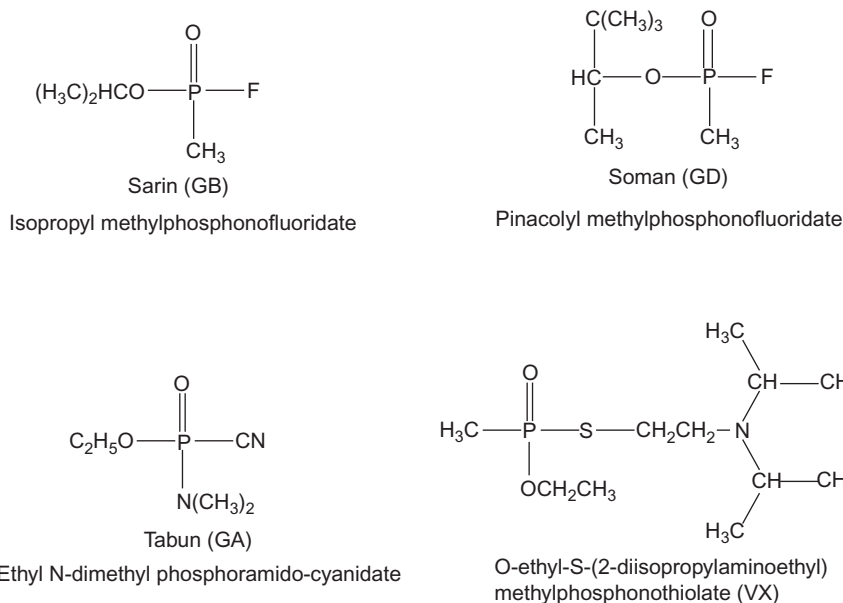


FIGURE 45.3 Chemical structures of OP nerve agents.

acyl-transfer, where water is the acceptor molecule to which the substrate acyl moiety is transferred. A serine oxygen of the active site gorge in ChEs carries out a nucleophilic attack on the electrophilic carbon of the carbonyl group of ACh, resulting in an acetylated enzyme intermediate and the release of choline. Deacetylation occurs when an attacking water molecule (hydroxyl ion) acts as a more effective nucleophile, thereby releasing acetate. The molecular interactions between OPs

and AChE (Figure 45.6) have been studied in much more detail than between CMs and AChE. The rates of hydrolysis and reactivation of AChE following carbamylation and phosphorylation of the active site appears to be markedly slower than for the hydrolysis of the acetylated enzyme. The turnover time for ACh is of the order of  $\sim 150 \mu\text{sec}$ , whereas the carbamylated enzyme  $t_{1/2}$  for hydrolysis is substantially slower ( $\sim 15\text{--}30 \text{ min}$ ). The phosphorylated enzyme is highly



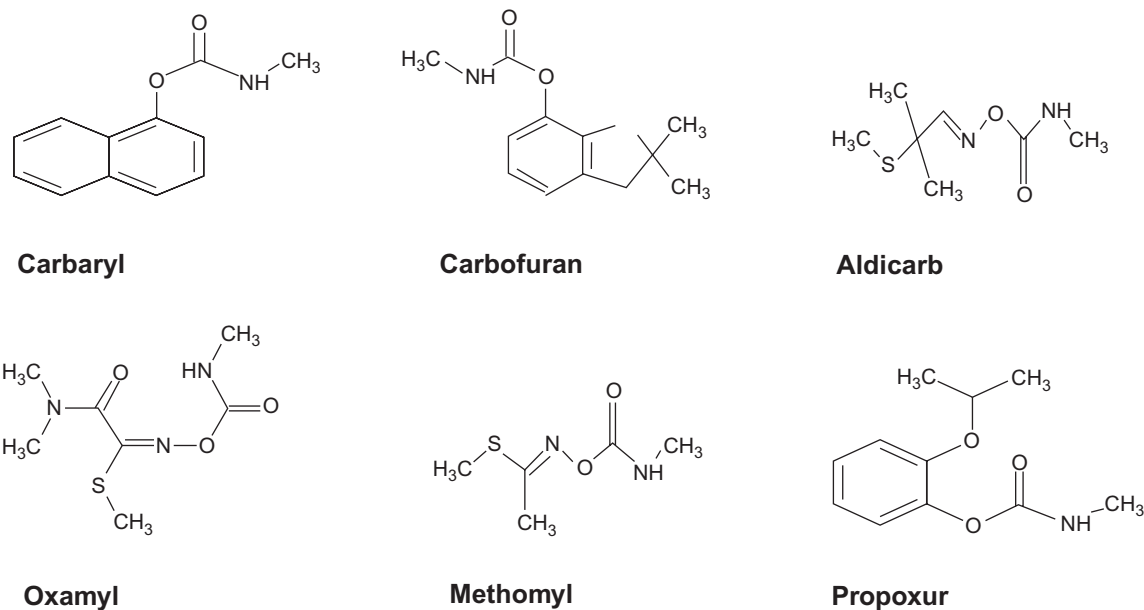
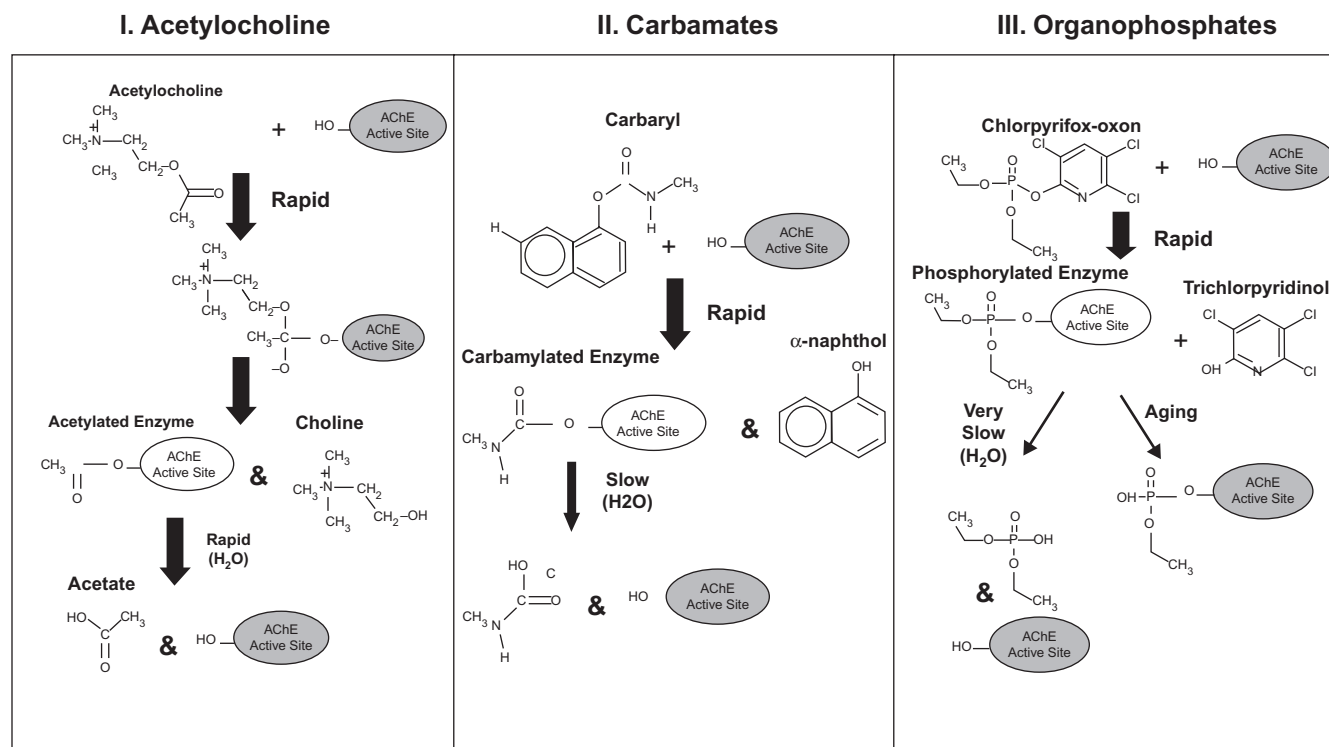


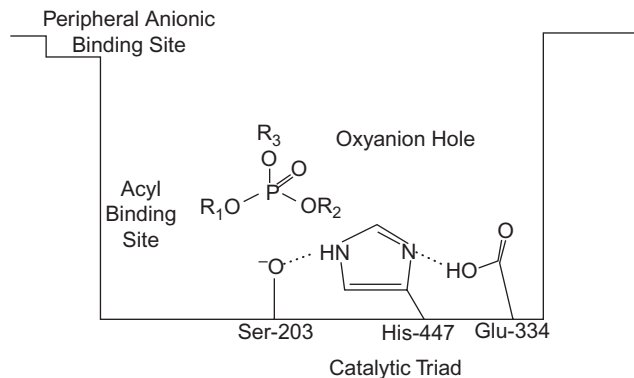
FIGURE 45.4 Chemical structures of commonly used CM pesticides.

TABLE 45.3 A brief chemical description of commonly used CM pesticides

Chemical name	Chemical name	Molecular weight	Oral LD <sub>50</sub> in rat (mg/kg)	Dermal LD <sub>50</sub> in rabbit (mg/kg)
Aldicarb	(1E)-2-methyl-2-(methylthio)propanal O-[(methyl-amino)carbonyl] oxime	190.26	0.9	5
Aminocarb	4-(dimethylamino)-3-methylphenyl methylcarbamate	208.26	30	275
Bendiocarb	2,2-dimethyl-1,3-benzodioxol-4-yl methylcarbamate	223.23	34	566
Benfuracarb	2,3-dihydro-2,2-dimethyl-7-benzofuranyl N-[n[2-(ethylcarbonly) ethyl] -N-isopropyl sulfenamoyl]-N-methylcarbamate	410.53	138	>2000
BPMC	2-sec-butylphenyl N-methylcarbamate	422.87	340	4200
Carbaryl	1-naphthyl methylcarbamate	201.22	307	2000
Carbofuran	2,2-dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate	221.25	8	2550
Carbosulfan	2,3-dihydro-2,2-dimethyl-7-benzofuranyl-[(di-butylamino)thio] methyl carbamate	380.55	209	>2000
Croneton	2-[(ethylthio)methyl]phenyl methylcarbamate	225.31	200	1000
Fenoxycarb	ethyl [2-(4-phen-oxyphenoxy)ethyl] carbamate	301.34	10,000	2000
Isoprocab	2-isopropylphenyl methylcarbamate	193.24	450	—
Methiocarb	3,5-dimethyl-4-(methylthio)phenyl methylcarbamate	225.31	15	2000
Methomyl	methyl (1E)-N-[(methylamino) carbonyl]oxy} ethanimidothioate	162.21	17	5000
Metolcarb	3-methylphenyl methylcarbamate	165	268	—
Mexacarbate	4-(dimethylamino)-3,5-dimethylphenyl methylcarbamate	222.28	15	5000
Oxamyl	methyl 2-(dimethyl-amino)-N-[(methyl-amino)carbonyl]oxy-2-oxoethan-imidothioate	219.26	5	710
Pirimicarb	2-(dimethylamino)-5,6-dimethyl-pyrimidin-4-yl dimethylcarbamate	238.29	147	>500
Promecarb	3-isopropyl-5-methylphenyl methylcarbamate	207.27	61	>1000
Propoxur	2-isopropoxyphenyl methylcarbamate	209.24	95	>1000
Trimethacarb	3,4,5-trimethylphenyl methylcarbamate	193.24	125	>2000
XMC	3,5-dimethylphenyl methylcarbamate	179.22	542	—
Xylylcarb	3,4-dimethylphenyl methylcarbamate	179.22	384	—



**FIGURE 45.5** Interaction of acetylcholine (I), the carbamate carbaryl (II), and the organophosphate chlorpyrifos-oxon (III) with the active site of acetylcholinesterase (AChE). The general rate of bound AChE hydrolysis is ACh > carbaryl > chlorpyrifos-oxon (Timchalk, 2006).



**FIGURE 45.6** Schematic drawing of the active site gorge of AChE, with the entry of an OP molecule. R<sub>1</sub> and R<sub>2</sub> on the OP are usually identical alkyl chains, whereas R<sub>3</sub> is the leaving group. The catalytic triad consists of Ser203, His447 and Glu334. The acyl binding site is likely important in positioning the inhibitor for the nucleophilic attack from Ser203 (Ordentlich *et al.*, 1996), whereas the oxyanion hole may polarize the P=O bond, thereby facilitating the nucleophilic attack (Ordentlich *et al.*, 1998). Binding of ligand to the peripheral anionic site can lead to inhibition or activation. Additionally, the peripheral anionic site plays an important role in the stereoselectivity of AChE toward methylphosphonates (Ordentlich *et al.*, 2004). Adapted from Sultatos (2006).

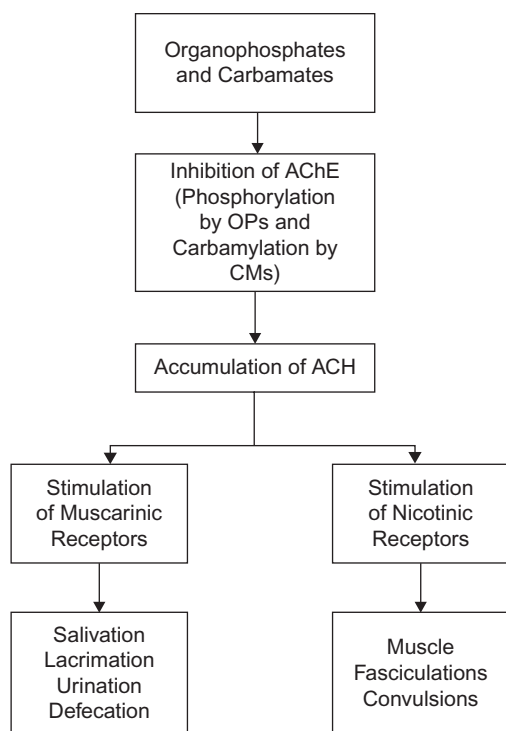
stable ( $t_{1/2}$  ~days), and further dealkylation of the phosphorylation group produces an “aged” AChE that is irreversibly inhibited (Taylor, 2006; Timchalk, 2006). In general, OPs and CMs are considered as irreversible

and reversible AChE inhibitors, respectively. Details of cholinesterases (ChEs), interaction of OPs and CMs with ChEs, and reactivation/regeneration of ChEs, are described elsewhere (Radic and Taylor, 2006; Sultatos, 2006; Timchalk, 2006; Jokanovic, 2010; Gupta and Milatovic, 2011).

By now, it is established that OP/CM-induced seizures and lethality are also associated with non-cholinergic mechanisms, such as *N*-methyl-*D*-aspartate (NMDA) receptors, and adenosinergic, gamma-aminobutynergic (GABA-ergic), and the monoaminergic systems (Gupta *et al.*, 2007; Dekundy and Kaminski, 2010; Myhrer, 2010; Gupta and Milatovic, 2011). Furthermore, the persistence of excitotoxicity for more than an hour can lead to oxidative and nitrosative stress, neuroinflammation and neurodegeneration in cortex, amygdala and hippocampus, which are the areas of brain primarily involved in initiation and propagation of convulsions and seizures.

## TOXICITY

Most animal poisoning cases in the field are acute in nature. Onset of clinical signs usually occurs within 15min to 1h, followed by signs of maximal severity. However, timings of maximal severity signs tend to



**FIGURE 45.7** Important steps involved in mechanism of toxicity of OPs and CMs.

vary depending upon the OP/CM compound and its dose, and species. For example, onset of clinical signs is delayed with chlorpyrifos (Dursban) and dimethoate (Rogor). Clinical signs observed in poisoned animals can be divided into local and systemic effects. The local effects involve the eyes and the lungs, owing to their exposure to vapors or droplets of the insecticides. These effects, however, are of significance in the case of animals only when exposure is via spraying. The systemic effects are primarily on the brain, skeletal muscles, lungs, heart and other organs.

The clinical signs can also be classified as muscarinic, nicotinic and central. Figure 45.7 describes the sequence of events involved in OP/CM toxicity. Muscarinic receptor-associated effects are manifested by vomiting, abdominal pain, salivation, lacrimation, urination, diarrhea (SLUD), miosis (pinpoint pupils), tracheobronchial secretion, lung edema and cyanosis. The nicotinic receptor-associated effects are produced on autonomic ganglia and skeletal muscles, and the affected animals show twitching of muscles, tremors, followed by convulsions and seizures. This condition may lead to paralysis. The central effects include apprehension and stimulation, followed by depression. The affected animals may also show restlessness, ataxia, stiffness of the neck and coma. Death occurs due to respiratory failure and cardiac arrest. It is important to mention that not all poisoned animals show all the clinical signs (as described

**TABLE 45.4** Normal acetylcholinesterase (AChE) activity in brain cortex of different species

Species	AChE ( $\mu\text{mol/g/h}$ )
Cattle	160
Swine	163
Sheep	170
Horse	124
Chicken	1098
Dog	200
Rat	255

above) with every OP or CM compound. Furthermore, at nonlethal doses, the signs of toxicity caused by each OP or CM can vary widely. This can be due to the fact that each OP or CM reacts not only with AChE but also with other targets, such as butyrylcholinesterase, carboxylesterases, neuropathy target esterase (NTE), acylpeptide hydrolase, arylformamidase, proteases, trypsin, chymotrypsin, cannabinoid CB1 receptor, albumin and many other enzymes, receptors and proteins (Gupta, 2004; Lockridge and Schopfer, 2006; Ruark *et al.*, 2011). While surviving animals usually recover within 3 to 6 h with CMs and within 24 h with OPs, animals exposed to OP nerve agents may show signs of toxicity for days.

Poisoning cases of OP or CM are usually diagnosed based on clinical signs and quantified levels of AChE inhibition in blood from a live animal and brain from a dead animal. Inhibition of AChE activity >70% is considered a positive case of poisoning. It should be noted that great species variability exists in normal values of AChE activity (Table 45.4). In addition, there is more than a six-fold variability in AChE activity in different brain areas with preference given to cortex and not the striatum for AChE analysis (Gupta, 2004). Therefore, interpretation should be made with great caution. Residue analysis of an insecticide and/or its metabolite(s), and by confirmation with GC/MS or LC/MS, seems an ideal approach for diagnosis.

## TREATMENT OF ACUTE POISONING

Before instituting antidotal therapy, monogastric animals, such as the dog, should be given gastric lavage. Animals of any species can be given activated charcoal to stop further absorption of insecticides. Animals should be washed thoroughly with water if they are exposed to insecticides dermally. Intravenous fluid therapy is always beneficial.

In the case of OP poisoning, antidotal treatment requires the combined use of atropine sulfate and pyridine-2-aldoxime methochloride (2-PAM). Atropine sulfate

acts by blocking the muscarinic receptors from ACh. In ruminants, one-fourth of the total recommended dose (0.5 mg/kg) can be given as a slow IV injection, and the remainder through IM or SC injection (Gupta, 1984). The total dose of atropine sulfate for an average size horse is about 65 mg, and for a dog is about 2 mg. Atropine sulfate treatment can be repeated at an interval of every hour until all hypersecretory signs have subsided. 2-PAM reactivates the AChE inhibited by OPs. The recommended therapeutic dose of 2-PAM is 20 mg/kg, IV. The injection of 2-PAM can be repeated once after 1 h at half of its initial dose. Care should be taken that only a freshly prepared solution of 2-PAM be used. It needs to be emphasized that the combined therapy of atropine sulfate and 2-PAM is superior to any other treatment till today in the case of OP poisoning. Although many other oximes have been tested against many OPs, none has been proven to be better than 2-PAM. Furthermore, the depressant drugs, such as morphine and barbiturates, are contraindicated, since they aggravate the condition. Diazepam without atropine sulfate also accentuates the toxicity of OPs.

Unlike with OP poisoning, 2-PAM and other oximes are ineffective in CM poisoning cases. In fact, in the case of some carbamates, such as carbaryl and carbofuran, 2-PAM therapy accentuates the toxicity. Some anticonvulsant drugs, such as barbiturates and diazepam, also aggravate the toxicity of CMs. Therefore, atropine sulfate, with doses as described for OPs, is the only preferred antidote. However, when the animals are exposed to very high doses of carbamates, atropine sulfate does not appear to be a life-saving antidote.

## OP-INDUCED INTERMEDIATE SYNDROME

OP insecticide-induced intermediate syndrome (IMS) was reported for the first time in human patients in Sri Lanka in 1987 (Senanayake and Karalliedde, 1987). The observations were made in ten patients who presented 24–96 h after acute cholinergic crisis from exposure to methamidophos, fenthion, dimethoate and monocrotophos. This syndrome has also been diagnosed in OP-poisoned patients in South Africa (1989), Turkey (1990), Belgium (1992), the United States (1992), Venezuela (1998), France (2000) and in many other countries. To date, OPs that are known to cause IMS include bromophos, chlorpyrifos, diazinon, dicrotophos, dimethoate, disulfoton, fenthion, malathion, methamidophos, methyl parathion, monocrotophos, omethoate, parathion, phosmet and trichlorfon. IMS is usually observed in individuals who have ingested a massive dose of an OP insecticide either accidentally or in a

suicide attempt. In 2005, a carbamate insecticide carbofuran was also demonstrated to cause IMS in patients accidentally or intentionally exposed to large doses of this insecticide (Paul and Mannathukkaran, 2005). IMS is clearly a separate clinical entity from acute toxicity and delayed polyneuropathy. Clinically, IMS is characterized by acute paralysis and weakness in the areas of several cranial motor nerves, neck flexors and facial, extraocular, palatal, nuchal, proximal limb and respiratory muscles 24–96 h after poisoning. Generalized weakness, depressed deep tendon reflexes, ptosis and diplopia are also evident. These symptoms may last for several days or weeks depending on the OP involved. A similar syndrome has also been observed in dogs and cats poisoned maliciously or accidentally with massive doses of certain OPs. It should be noted that despite severe AChE inhibition, muscle fasciculations and muscarinic receptor-associated hypersecretory activities are absent.

Although the exact mechanism involved in pathogenesis of IMS is unclear, studies suggest that decrease of AChE and nicotinic ACh receptor mRNA expression occur after oral poisoning with disulfoton in rats. Involvement of oxidative stress is also suggested (Dandapani *et al.*, 2003). Based on electromyographic (EMG) findings from OP-poisoned patients and experimental studies on laboratory animals, it has been found that the defect in IMS is at the neuromuscular endplate and postsynaptic level, but the effects of neural and central components in producing muscular weakness have not been ruled out. It seems clear that some OPs are greatly distributed to muscles and have higher affinity for nicotinic ACh receptors. Currently, very little is known about the type of damage at the motor endplate or about risk factors contributing to its development. There is no specific treatment, and therapy relies upon atropine sulfate and 2-PAM. The administration of atropine sulfate and 2-PAM should be continued for a long period, even if efficacy of these drugs on the development of IMS appears to be limited. For further details about IMS, readers are referred to Gupta (2005) and De Bleeker (2006).

## CHRONIC TOXICITY

Chronic toxicity is a major concern with OP pesticides. OP compounds that produce delayed neurotoxic effects are esters of phosphorus-containing acids. Over 40 years ago, tri-*o*-cresyl phosphate (TOCP) was known to produce delayed neurotoxic effects in man and chicken, characterized by ataxia and weakness of the limbs, developing 10 to 14 days after exposure (Johnson, 1969). This syndrome was called OP-induced delayed neuropathy (OPIDN). In recent literature, the syndrome



has been renamed OP-induced delayed polyneuropathy (OPIDP). OPIDP is characterized by distal degeneration of long and large-diameter motor and sensory axons of both peripheral nerves and spinal cord. Among all animal species the hen appears to be the most sensitive and therefore used as an animal model. TOCP and certain other compounds have minimal or no anti-AChE property; however, they cause phosphorylation and aging (dealkylation) of a protein in neurons called neuropathy target esterase (NTE), and subsequently lead to OPIDP. Studies on the sensitivity of the target enzymes of a variety of OPs showed that the comparative inhibitory power of OPs against hen AChE and NTE *in vitro* correlates with their comparative effects *in vivo* (i.e., delayed neuropathy or death). The relationship between the degree of NTE inhibition and the severity of OPIDP changes according to the compound involved and the source of NTE. For example, certain compounds cause OPIDP with a minimum of 70% NTE inhibition, while others require almost complete inhibition to cause OPIDP. Inhibition of peripheral nerve NTE is required to develop OPIDP and no clinical deficits appear if only brain NTE is inhibited. For neuropathy to occur, a second reaction called “aging” of NTE must take place and this involves cleavage of the lateral side chain from the phosphorylated NTE. This reaction is called aging because it is a slow progressive process and the product is no longer responsive to nucleophilic reactivating agents, such as oximes. The cascade of events from NTE inhibition/aging to impairment of retrograde axonal transport and axonal degeneration is yet to be explained (Moretto and Lotti, 2006). Today, many compounds, such as DFP, *N,N'*-diisopropyl phosphorodiamidic fluoride (mipafox), tetraethyl pyrophosphate (TEPP), parathion, *o*-cresyl saligenin phosphate and haloxon, are known to produce this syndrome. Some OPs as well as non-OP inhibitors (such as carbamates and sulfonyl fluorides) also covalently react with NTE but cannot undergo the aging reaction. As a result, these inhibitors do not cause OPIDP, and when given to experimental animals prior to neuropathic OP, may protect from OPIDP when they occupy at least 30% of the NTE active site. For the details of OPIDP syndrome, readers are referred to Morreto and Lotti (2006) and Wu and Chang (2010). Treatment of this syndrome is symptomatic.

## TOLERANCE DEVELOPMENT

Tolerance development to the toxicity of OPs was noted more than 50 years ago. Following prolonged exposure to an OP, the physiological effects often diminish more than expected from the degree of AChE inhibition or

repeated additions of OP give lower responses with time. Tolerance to AChE inhibiting OPs (such as DFP, disulfoton, methyl parathion and others) has been observed using different forms of administration and in different species, such as mice, rats, guinea pig and man (Fonnum and Sterri, 1981, 2006; Gupta and Dettbarn, 1986; Gupta *et al.*, 1986; Gupta, 2004).

Tolerance to OP toxicity can develop in several ways. Most often, it occurs due to receptor changes either in the number of receptors or by decreased affinity of the receptor molecule. However, it can also occur due to the presence of other proteins that can bind or inactivate the inhibitor and thereby make it less readily available. Some of the examples for binding to the OPs are carboxylesterases (CarbEs), butyrylcholinesterases (BuChEs) or other binding proteins such as albumin (Sogorb and Vilanova, 2010). In addition, tolerance can be achieved through more rapid metabolism of the OP compounds by OP-hydrolyzing enzymes such as paraoxonases (PONs) and somanases (Fonnum and Sterri, 2006).

ACh receptors (both mAChRs and nAChRs) are involved in the development of tolerance to OP toxicity. Treatment with a cholinergic agonist for a prolonged time leads to a decrease in the muscarinic ACh receptors (mAChRs). This is common for G protein-linked receptors. In some studies, OPs have been found to cause decrease in the numbers of mAChRs in the brain, while in others both the number of mAChRs and the affinity to the ligand in ileum and striatum.

Significant reductions in nAChRs numbers ( $B_{max}$ ), without change in affinity ( $K_D$ ), have been found in brain of tolerant rats treated with disulfoton (Costa and Murphy, 1983) and in skeletal muscle of rats treated with DFP (Gupta *et al.*, 1986; Gupta and Dettbarn, 1986). In tolerant rats, significant recovery of CarbEs and BuChEs has also been found. In essence, tolerance development following subchronic or chronic treatment with AChE inhibiting OPs occurs through multiple mechanisms.

## CONCLUSIONS AND FUTURE DIRECTIONS

OPs and CMs constitute a large number of chemicals that are used in agriculture primarily as insecticides and in veterinary medicine as parasiticides. These chemicals exert a broad range of toxic effects, varying from mild effects such as salivation and tremors to serious effects such as convulsions, seizures, paralysis and death. Basically, OPs and CMs are neurotoxicants, but directly or indirectly several vital organs are affected. These chemicals produce a variety of toxicological effects on the central nervous system, peripheral nervous system

and cardiovascular, pulmonary, ocular, neurobehavioral, immunological, reproductive, placental, cutaneous and other body systems. In addition, these insecticides cause neurodegeneration, oxidative stress, endocrine disruption and many other complications. In general, OPs produce more serious and lingering health effects than CMs. These devastating effects, including a complex syndrome like OPIDP, are yet to be mechanistically characterized and fully defined. It is expected that newer compounds of both OP and CM classes with greater selective toxicity as well as improved antidotes effective in patients with OPIDP or against CMs will be developed.

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# Organochlorines

Steve M. Ensley

## INTRODUCTION

Many methods have been used and developed to control or eliminate insects and other plant and animal pests that have threatened man and his food supply. A few examples of chemicals used in the past to control insects are sulfur (used before 1000 BC and is still used today), vinegar, whale oil and arsenic (used in the 1500s), nicotine (used in the 1600s), rotenone from the plant *Derris elliptica*, pyrethrum from *Chrysanthemum cinerariaefolium*, copper arsenate (used in the 1800s) and calcium and lead arsenate (used in the early 1900s). There were major changes in the development and use of insecticides in the 1930s when the synthetic organic chemical industry began developing compounds to control insects. Chlorinated compounds, cyclodienes such as aldrin and dieldrin, used as insecticides, became available for use in the 1940s. Dichlorodiphenyltrichloroethane (DDT) became available during WWII and was used extensively as an insecticide worldwide. One of the reasons that organochlorines were effective insecticides was their ability to persist in the environment (Leonard *et al.*, 1999; Hites *et al.*, 2004; Hoekstra *et al.*, 2005). Because of that persistence, most have been eliminated from use today. Lindane (gamma-hexachlorocyclohexane) and endosulfan are the most biodegradable organochlorines and are still used today.

The diphenyl aliphatic organochlorines, such as DDT, affect the peripheral nerves and brain by slowing sodium ( $\text{Na}^+$ ) influx and inhibiting potassium ( $\text{K}^+$ ) outflow. This results in excess intracellular  $\text{K}^+$  in the neuron, which partially depolarizes the cell.

In the 1980s, the mechanism of toxicity for the cyclodiene organochlorine insecticides was determined. These

compounds were found to be non-competitive antagonists acting on the chloride ion channel of the gamma-aminobutyric acid A (GABA) receptor.

## BACKGROUND

Even though DDT was first synthesized by Othmar Zeidler in 1874, it was another 40 years before this compound was used as an insecticide. Paul Mueller, a Swiss chemist, rediscovered DDT in 1939 while investigating insecticides for use against clothes moths and carpet beetles. Mueller won the Noble Prize in 1948 for this work. "This pleasant smelling, greasy white powder (DDT) has had an influence on human ecology perhaps unmatched by any other chemical discovery including gunpowder, sulfanilamide, penicillin, and plutonium" (Metcalf, 1973).

The insecticidal properties of technical HCH (t-hexachlorocyclohexane; commonly known as benzene hexachloride) and the first cyclodiene insecticides (e.g., aldrin, dieldrin, chlordane) were discovered as a result of the commercial interest in new uses for chlorine and hydrocarbons such as cyclopentadiene and benzene. The first use of the chlorinated hydrocarbons was for dielectrics and as fire retardants. The use of these compounds as insecticides occurred when benzene was added to liquid chlorine in the field and it was noted that the product killed insects.

As with many insecticides there are many unintended secondary targets for every insecticide (Uzoukwu and Sleight, 1972; Furie and Trubowitz, 1976; Hathway, 1977). The following is a brief discussion about the



organochlorines and some of the intermediates used in their production. Hexachlorocyclopentadiene, a raw material used in manufacturing chemicals, was known to be stable and was found to react easily with cyclopentadiene in a Diels-Alder reaction, which leads to the production of chlordane. It was discovered later to react with norbornadiene (a bicyclic hydrocarbon) to produce aldrin. Allylic chlorination of chlordane produces heptachlor. The intermediate hexachloronorbornadiene (HCNB) reacts with cyclopentadiene to produce isodrin and after epoxidation, dieldrin and endrin are produced. Technical hexachlorocyclohexane (t-HCH) can be used to produce the gamma isomer lindane. One of the problems with the production of lindane is the inefficiency of the process; for every ton of lindane produced, 8–10 tons of the inactive alpha and beta isomers are formed. Because of the widespread use of t-HCH, the environment has become contaminated with the inactive alpha and beta-HCH isomers.

Overcoming the problem of insect resistance to the organochlorines has also been associated with toxicity. A major mechanism of insect resistance to DDT was found to be enzymatic dechlorination of DDT to dichlorodiphenyldichloroethylene (DDE) (Bonner and Yarbrough, 1988). While working to overcome resistance to DDT, it was discovered that certain nontoxic DDT analogs and other compounds suppressed resistance when co-applied with DDT. Toxicity to parent compounds as well as congeners has been associated with use of the organochlorines.

The beginning of the science of toxicology can be traced to the problems associated with use of DDT and the subsequent impact on man and the environment. Rachel Carson's book *Silent Spring* brought the problems associated with the use of DDT to national attention in 1962. Organochlorines continue to be an environmental contaminant as evident in this 2010 study (Ding *et al.*, 2010).

The structures of various organochlorine insecticides are shown in Figure 46.1.

## PHARMACOKINETICS/ TOXICOKINETICS

Organochlorine insecticides can be absorbed orally and topically, with absorption being rapid due to the lipid solubility of these compounds (Buck *et al.*, 1976; Marth *et al.*, 1989). Organochlorine insecticides are not highly volatile, so inhalation is not a normal route of exposure (Jaeger *et al.*, 1973). Distribution is to the liver, kidney, brain and adipose tissue (Buck and Van Note, 1968; Buck, 1970; Booth and McDowell, 1975). The acute toxicity caused is of concern, but bioaccumulation from chronic

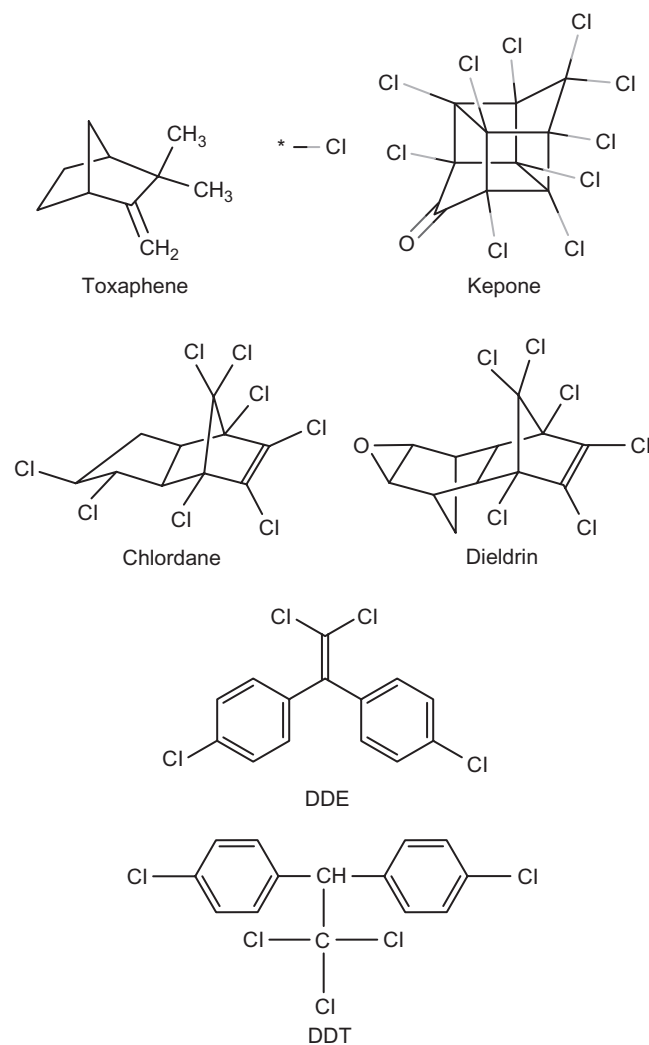


FIGURE 46.1 Structures of common organochlorines.

exposures is equally important (Starr and Clifford, 1972). Chlorinated hydrocarbons are highly lipid soluble and persist in the environment; as a result bioaccumulation occurs in the food chain from the environment to animals and humans (Mount *et al.*, 1980; Oehme, 1991; Safe and Krishnan, 1995; Watanabe *et al.*, 1999; Backer *et al.*, 2001; Smith and Gangolli, 2002; Harris *et al.*, 2005).

As with all xenobiotics, the toxicity of the organochlorines is related to absorption, distribution, metabolism and elimination (Jaeger *et al.*, 1975; Beasley *et al.*, 1994). The diphenyl aliphatics, such as DDT, are dechlorinated by mixed function oxidases (MFOs). Aryl hydrocarbons, like paradichlorobenzene, undergo glucuronidation and sulfation. The cyclodiene insecticides, such as endrin, are rapidly converted to epoxides by MFOs. Methoxychlor is rapidly eliminated compared to DDT by dechlorination and oxidation. The intermediates of organochlorine insecticide production may be more toxic than the parent compound.

The major excretory route of organochlorines is from bile into the digestive tract, and as a result enterohepatic recycling can occur. Metabolites are also lipophilic, will move into adipose tissue and are released slowly from lipid depot storage (Sell *et al.*, 1977). The half-life of some diphenyl aliphatics, such as DDT and the cyclodienes, may range from days to years (Council for Agricultural Science and Technology, 1974). Elimination can sometimes be explained by a two-compartment model, where the first phase is rapid elimination and the second is prolonged.

## MECHANISM OF ACTION

There are at least two different mechanisms of action for organochlorine insecticides (Shankland, 1982; Narahashi, 1987; Osweiler, 1996). DDT-type organochlorine (dichlorodiphenylethanes) insecticides affect the peripheral nerves and brain by slowing sodium ( $\text{Na}^+$ ) influx and inhibiting potassium ( $\text{K}^+$ ) efflux. This results in excess intracellular  $\text{K}^+$  in the neuron, which partially depolarizes the cell. The threshold for another action potential is decreased, resulting in premature depolarization of the neuron.

The aryl hydrocarbons and cyclodienes, in addition to decreasing action potentials, may inhibit the postsynaptic binding of GABA (Bloomquist and Soderlund, 1985; Lummis *et al.*, 1990; French-Constant, 1993; Hahn, 1998; Carr, *et al.*, 1999). The cyclodiene organochlorine insecticides act by competitive inhibition of the binding of GABA at its receptor, causing stimulation of the neuron, as described below (Joy, 1976, 1982; Gandolfi *et al.*, 1984).

GABA is a neurotransmitter in the mammalian and insect central nervous system and the inhibitory neurotransmitter for insects at the neuromuscular junction.  $\text{GABA}_\text{A}$  receptors, present in mammalian and insect synapse, are ligand gated chloride ion channels. In mammals,  $\text{GABA}_\text{B}$  receptors are coupled to calcium and potassium channels and the action of GABA is mediated by G-proteins.  $\text{GABA}_\text{B}$  receptors are not important in insect physiology. When GABA is released in the synapse it diffuses to the presynaptic terminal of another nerve, where it binds to a  $\text{GABA}_\text{A}$  receptor. This causes chloride ions to enter the synapse resulting in hyperpolarization of the terminal and inhibition of release of other neurotransmitters. Because of this inhibition, post-synaptic stimulation of other nerves by other transmitters (e.g., acetylcholine) is reduced. When GABA is inhibited, there is no synaptic down-regulation and other neurotransmitters can be released in excess. The inhibitory mechanism of GABA explains the cholinergic effects (over stimulation by acetylcholine) of dieldrin and lindane on some species.

TABLE 46.1 Toxicity data for some organochlorines

Compound	Rat acute oral $\text{LD}_{50}$ (mg/kg)	Rabbit dermal $\text{LD}_{50}$ (mg/kg)
Lindane	76–190	500
Aldrin	39–60	65
Dieldrin	40	65
Endrin	3	12
Chlordane	283–1100	580
Endosulfan	18–76	74
Mirex	235–>3000	800
Kepone	95–125	345
Toxaphene	40–127	600

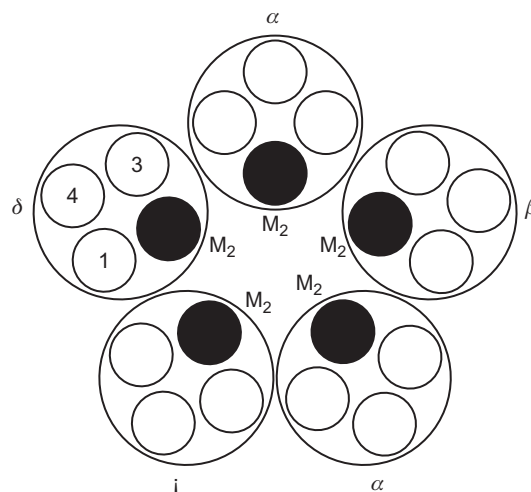


FIGURE 46.2 Illustration of the  $\text{GABA}_\text{A}$  receptor of the mammalian brain. The  $\text{M}_2$  segments form the chloride ion channel (McDonald and Olsen, 1994).

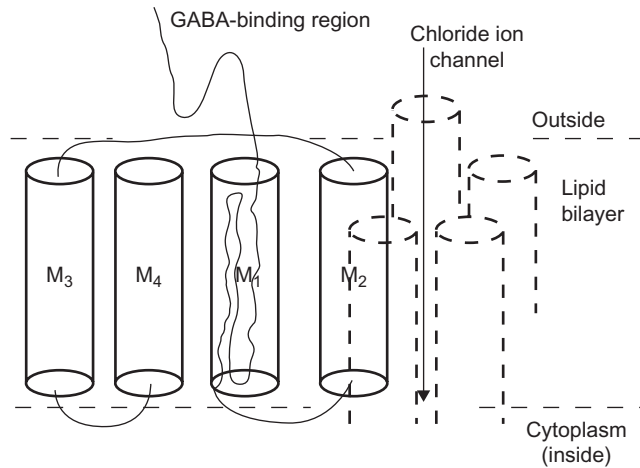
## TOXICITY

Cats are the most sensitive species to organochlorine insecticides, with the  $\text{LD}_{50}$  for endrin in cats of 3–6 mg/kg. The cyclodiene organochlorine insecticides cause more seizure activity and have a lower  $\text{LD}_{50}$  than the DDT-type insecticides in most species.

The oral  $\text{LD}_{50}$  for DDT in rats is 113–2500 mg/kg and the IV  $\text{LD}_{50}$  is 47 mg/kg. In humans, toxic signs from oral exposure to organochlorines can be observed at 10 mg/kg.

The acute toxicity to rats and the housefly of other chlorinated insecticides is shown in Table 46.1.

A description of the  $\text{GABA}_\text{A}$  receptor in the human brain is presented in Figures 46.2 and 46.3 to clearly describe the chloride ion channel. The  $\text{GABA}_\text{A}$  receptor of the human brain consists of four or five 50–60 kDa glycoprotein subunits, each of which contains four ( $\text{M}_1$ – $\text{M}_4$ ) hydrophobic domains. The five  $\text{M}_2$  domains are arranged to form a 5.6 Å diameter ion channel.



**FIGURE 46.3** Illustration of the GABA<sub>A</sub> receptor of the mammalian brain (McDonald and Olsen, 1994).

In addition to the effects on the nervous system, DDT metabolites can inhibit the output of the adrenal gland by selective necrosis of the zona fasciculata and the zona reticularis.

Following an acute or chronic exposure, organochlorines can produce a variety of reproductive and developmental effects in animals as well as humans (Gupta, 2009; Malik *et al.*, 2011). Chronic exposure to DDT has been documented to cause eggshell thinning and reduced fertility in wild birds.

## TREATMENT

No specific antidotes for organochlorine insecticides are available (Osweiler, 1996). Detoxification is the most essential component of therapy for organochlorine toxicity. If dermal exposure has occurred, the animal should be thoroughly washed with a detergent and water to remove the insecticide so absorption ceases. The hair of heavily contaminated long haired animals should be clipped. Personnel treating animals should exercise caution and prevent themselves from becoming contaminated with the insecticide by wearing gloves, aprons or raincoats.

For oral exposure to organochlorines, activated charcoal (1–2 g/kg) should be administered orally. An alternative but less effective treatment is mineral oil. The insecticide can dissolve in mineral oil, which decreases its absorption systemically. The approximate oral dose of mineral oil is 2–6 ml in cats, 5–15 ml in dogs and 1–3 liters in large animals. Charcoal or nonabsorbable oils are most effective when given within 4 hours of ingestion of the pesticide (Aslani, 1996).

General supportive care includes the use of antiseizure medications such as diazepam, phenobarbital or pentobarbital. Animals should be placed in a warm and comfortable area to minimize trauma when they are seizing. Animals recovering from organochlorine insecticide exposure may have to be monitored long term because organochlorines can persist in the body for months or years. The source of the exposure must be identified and removed to stop exposure. One decontamination strategy is to reduce feed intake so that the animal loses body fat, thereby reducing organochlorine residues in adipose tissue. Lactating animals rapidly eliminate organochlorine residues because the residues are excreted in milk. An additional treatment option in large animals is to feed activated charcoal (500–1000 g/day) to reduce enterohepatic recycling.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

The use of organochlorine insecticides is decreasing in the United States but they are still used worldwide. In the United States, with the ability of these compounds to persist in the environment and bioaccumulate, the organochlorine insecticides will continue to be an important toxicant for many years (Custer *et al.*, 2005).

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## Pyrethrins and pyrethroids

Steve M. Ensley

### INTRODUCTION

Pyrethrins are the insecticidal compounds obtained from the flowers of the plant *Tanacetum cinerariaefolium*, also called *Chrysanthemum cinerariaefolium* or *Pyrethrum cinerariaefolium*. Pyrethrum denotes extracts from the flowers that contain the active pyrethrin compounds (Proudfoot, 2005). The use of pyrethrum in insecticide preparations dates back to Persia, about 400 BC. Pyrethroids are synthetic analogs of pyrethrins. Because of stability problems with the natural pyrethrins, these insecticides were replaced by the more stable organophosphate and organochlorine insecticides developed after World War II (Valentine, 1990). As a result of the toxicity and environmental contamination associated with the organophosphate and organochlorine insecticides, interest in the use of pyrethrins and pyrethroids re-emerged in the 1970s. Pyrethrin and pyrethroid insecticides are effective against a variety of insect pests on companion animals and livestock, and are used on farms, in the home and garden and have many public health applications because of the safety associated with these compounds.

### BACKGROUND

There are six compounds that comprise the natural pyrethrins; pyrethrin I and II, jasmolin I and II and cinerin I and II. Synthetic pyrethroids have been developed because the natural pyrethrins tend to break down quickly when exposed to air, light and heat. The synthetic pyrethroids can be classified as first and second

generation. First generation pyrethroids are esters of chrysanthemic acid and an alcohol, having a furan ring and terminal side chain moieties. Second generation pyrethrins have 3-phenoxybenzyl alcohols derivatives in the alcohol moiety, and have had some of the terminal side chain moieties replaced with a dichlorovinyl or dibromovinyl substitute and aromatic rings. Addition of the alpha-cyano group to the 3-phenoxybenzyl alcohol group in the second generation pyrethroids has increased the insecticidal potency.

Pyrethrins cause hyperexcitability with very little cytotoxicity. The molecular targets of the pyrethrins and pyrethroids are similar in mammals and insects and include voltage-gated sodium, chloride and calcium channels, GABA-gated chloride channels, nicotinic receptors, membrane depolarization and intercellular gap junctions (Forshaw and Ray, 1990; Song and Narahashi, 1996a). Mammals are less susceptible to pyrethrin and pyrethroid toxicoses than insects primarily because they have a faster metabolic clearance, higher body temperatures and a lower affinity for the pyrethrins/pyrethroids (Song and Narahashi, 1996b) (Figure 47.1).

### PHARMACOKINETICS/ TOXICOKINETICS

Determination of the toxicity of pyrethroids *in vivo* is difficult because they have low water solubility, easily partition into lipids and will bind to plastics and glass. The reported toxicity of this class of insecticides has high variability.

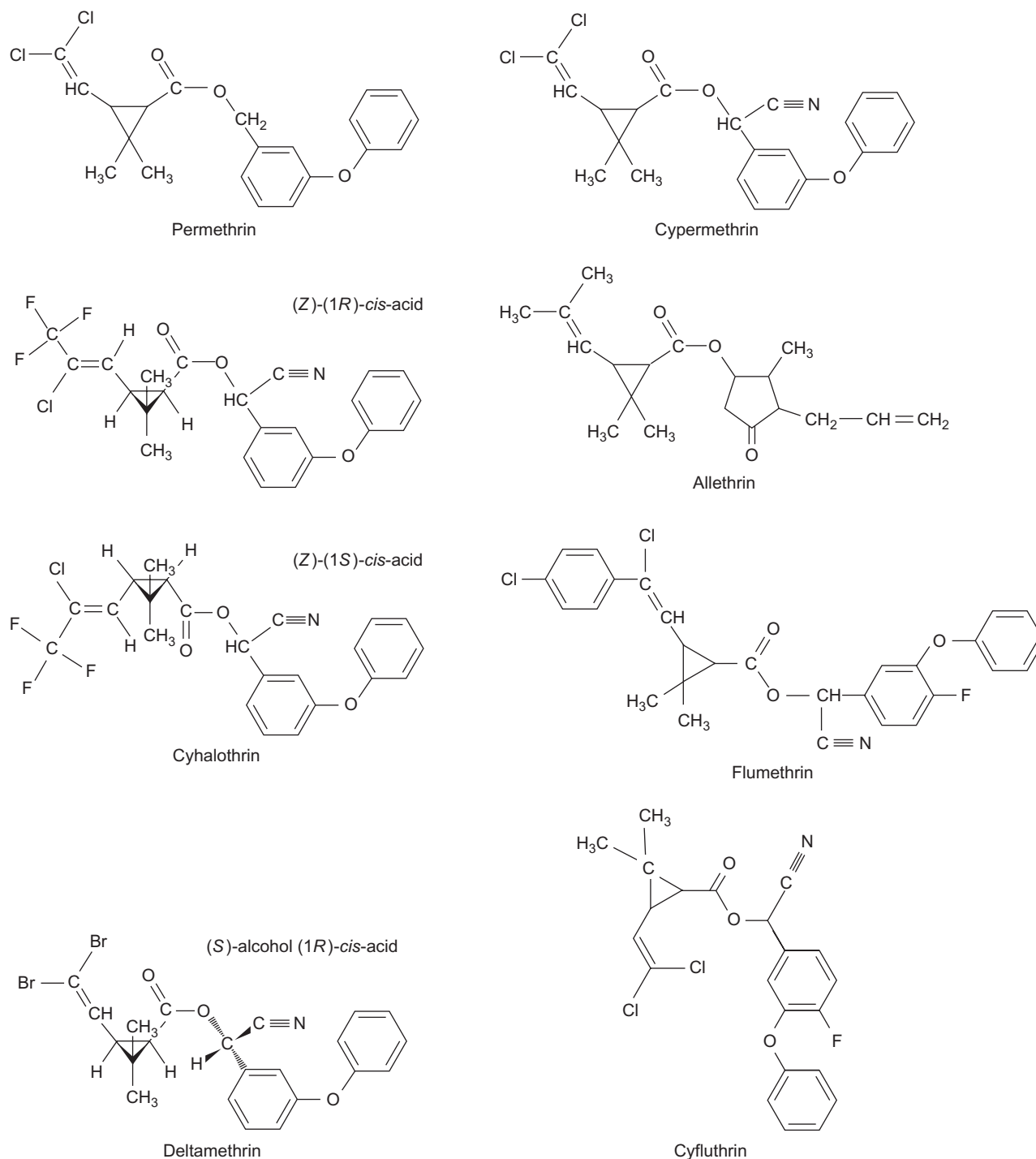


FIGURE 47.1 Structures of selected pyrethrins and pyrethroids.

Most pyrethrin and pyrethroid products are applied dermally in animals, but because of grooming, there can be oral and inhalation exposures, too. Less than 2% of topically applied pyrethrin and pyrethroid insecticides are absorbed dermally (Wollen *et al.*, 1992). One study confirmed that absorption of cypermethrin across human skin is minimal and peak excretion rates

after dermal application were not observed until 12 to 36 hours after dosing. Pyrethrins may be sequestered in the skin and slowly released into the systemic circulation (He *et al.*, 1989). Oral or inhalation exposure results in faster systemic exposure (Anadon *et al.*, 1996). Approximately 40–60% of an orally ingested dose is absorbed. When cypermethrin was administered orally

to six adult male volunteers, oral absorption ranged from 27 to 57% of the administered dose and peak excretion rates were measured in the urine between 8 and 24 hours after dosing. When adult males were exposed to cyfluthrin at  $160\mu\text{g}/\text{m}^3$ , 93% of the metabolites were excreted within the first 24 hours with peak excretion rates ranging from 0.5 to 3 hours.

Pyrethroids are lipophilic and will distribute to tissues with high lipid content such as fat and nervous tissue in addition to liver, kidney and milk. Recently, Kim *et al.* (2008) described the pharmacokinetics and tissue distribution of deltamethrin in adult rats following oral or intravenous administration. Utilizing a physiologically based toxicokinetic model, GI absorption of deltamethrin was rapid, but bioavailability was low. Deltamethrin in blood was largely present in plasma. A very small proportion of the absorbed doses reached or remained in the brain. Fat, skin and muscle ultimately accumulated large amounts of this highly lipophilic insecticide.

Pyrethroids and pyrethrins are rapidly hydrolyzed in the gastrointestinal tract. Once absorbed these compounds are metabolized by mixed function oxidases and esterases. Metabolism of the pyrethroids results in water soluble metabolites. Metabolism includes hydrolysis of the central ester bond, oxidation at several sites and conjugation with glycine, sulfate, glucuronide or glucosides. Cleavage of the ester bond results in substantial reduction in toxicity. The presence of the alpha-cyano group, as in type II pyrethroids, will decrease the rate of hydrolysis of the ester bond. Cleavage of the alpha-cyano group results in rapid conversion of the cyano group to thiocyanate.

Pyrethroids are eliminated by first order kinetics and most of the dose is eliminated in the first 12–24 hours after absorption. The primary routes of excretion are urinary and fecal, as a mix of parent compound and metabolites.

## MECHANISM OF ACTION

Pyrethroids primarily affect the sodium channel of cells, but chloride and calcium channels are also affected. Pyrethrins and pyrethroids slow the opening and closing of the sodium channels, resulting in excitation of the cell (Marban *et al.*, 1989; Conley and Brammar, 1999). The increase of sodium in the sodium channels results in a cell that is in a stable, hyperexcitable state. The duration of the sodium action potential is much longer for type II pyrethroids than for type I. Type I pyrethroids result in primarily repetitive charges with membrane depolarization predominating in type II pyrethroids. Paresthesia results from the direct action of pyrethroids on sensory

nerve endings, causing repetitive firing of these fibers. Less than 1% of sodium channels must be modified by pyrethroids to produce neurological signs. High concentrations of type II pyrethroids may also act on GABA-gated chloride channels (Bloomquist *et al.*, 1986).

Pyrethrins also affect the voltage-dependent chloride channels. These channels are found in the brain, nerve, muscle and salivary gland and control cell excitability. There are many different functional types of chloride channels in contrast to sodium channels. Most pyrethroid-sensitive channels belong to the Maxi chloride channel class. Maxi channels are activated by depolarization, have high conductance, are calcium independent and are activated by protein kinase C phosphorylation. Pyrethroids cause a decrease in the Maxi chloride channel current, which increases excitability of the cell just as the action of pyrethroids on the sodium channel.

The decreased sensitivity of mammals to this class of compounds compared to insects is due to several factors. Pyrethroids bind more strongly with the sodium channel at low temperatures than at high temperatures. Insects' ambient temperature is approximately  $25^\circ\text{C}$  compared to mammals at  $37^\circ\text{C}$ . Mammalian sodium channels are at least 1000 times less sensitive to pyrethroids than insect sodium channels. Mammalian sodium channels recover much more quickly from depolarization than do insect sodium channels and are much more likely to detoxify pyrethroids before they reach their target site than are insects.

Pyrethroids cause a phenomenon in insects called "knockdown" (Narahashi, 1985). Knockdown is caused by inhibiting the cell but not causing a lethal effect. This is caused from the ability of the sodium channels to retain many of the normal functions, such as selectivity for sodium ions and conductance after exposure to pyrethroids. After exposure to moderate doses of pyrethroids, cells function in a new state of hyperexcitability. If the level of sodium in the ion channel does not exceed the ability of the sodium pump to remove it, the cell continues to function normally. High concentrations of pyrethroids or hyperactivity beyond what the cell can sustain will cause depolarization and conduction block. The pyrethroids that hold the sodium channel open the longest will cause the greatest amount of depolarization.

There is marked stereospecificity of the action of pyrethroids on the sodium channel; some isomers are more toxic than others (Soderlund, 1985, 2012; Meacham *et al.*, 2008). The *cis* isomers are usually more toxic than the *trans* isomers. As an example, the 1*R* and 1*S* *cis* isomers bind competitively to one site, and the 1*R* and 1*S* *trans* isomers bind noncompetitively to another (Narahashi, 1986). In mammals the 1*R* isomers are active and the 1*S* isomers inactive, making the 1*S* isomers non-toxic. Deltamethrin has been produced using stereospecificity to produce a high degree of selective toxicity. This is the reason that the toxicity of different batches of

pyrethroids can vary from batch to batch. The rat oral LD<sub>50</sub> of commercial permethrin can vary from 430 to 8900mg/kg, with toxicity depending on the amount of *cis* isomer present in the batch.

TOXICITY

Dermal exposure to pyrethroids is most common (Osweiler, 1996). In humans, the bioavailability of pyrethroids applied dermally is approximately 1%. Absorption after oral exposure in humans is 36%, mostly from the stomach. Once absorbed, the pyrethroids are rapidly distributed due to their lipophilicity. Systemic distribution produces effects that can be difficult to control and may be confused with poisoning by other pesticides, such as organophosphates which also cause increased salivation and hyperexcitability. Many pyrethroid formulations also contain solvents which can also cause toxicity. Cats are very sensitive to pyrethroid exposure (Meyer, 1999; Malik *et al.*, 2010).

The half-life of pyrethroids in general in plasma is in hours, while oral exposure can be equally short. Cyfluthrin has a plasma half-life of 19–86 minutes. Intravenous LD<sub>50</sub>s for pyrethroids range from 0.5 to 250mg/kg. The major neurotoxicity observed in adults with pyrethroid toxicity is acute toxicity with no chronic or cumulative toxicity being caused. The excitatory motor signs are generated at the spinal level.

Fish are highly sensitive to pyrethrin and pyrethroid products, and contamination of lakes, streams, ponds or any aquatic habitat should be avoided (Bradbury and Coats, 1986, 1989; Ansari and Kumar, 1988). Household exposure of fish can occur when the premise is sprayed or fogged with insecticides and the aquarium aerator is left on. The tank and aerator should be covered during use of insecticides and the home should be well ventilated before uncovering and starting the pump.

Most avian species are thought to be tolerant of pyrethrin and pyrethroid products but carriers or propellants in spray formulations may be hazardous (Bradbury and Coats, 1982). There is very little literature about pyrethrin or pyrethroid toxicity of exotic avian species, reptiles or lagomorphs.

Tables 47.1 and 47.2 reference the oral toxicity of some type I and II pyrethroids.

In dogs, cats and large animals the clinical signs are similar for both type I and II compounds. Clinical signs include salivation, vomiting, hyperexcitability, tremors, seizures, dyspnea, weakness, prostration and death (Murphy, 1996). In rats with type I toxicity there is an increased response to stimulation, muscle tremors, excitement and paralysis (Beasley *et al.*, 1994). These

TABLE 47.1 Toxicity of selected type I pyrethroids

Type I compounds	Oral LD <sub>50</sub> (mg/kg body wt.) in rat
Pyrethrin I	900
Allethrin	680
Tetramethrin	4640
Resmethrin	100
Permethrin	2000

TABLE 47.2 Toxicity of selected type II pyrethroids

Type II compounds	Oral LD <sub>50</sub> (mg/kg body wt.) in rat
Cypermethrin	500
Deltamethrin	31
Fenvalerate	450
Fluvalinate	1000

clinical signs can also be compatible with strychnine toxicities. Type II overexposure will cause increased salivation, weakness and choreoathetosis. The concomitant use of pyrethrins and pyrethroids with synergists such as piperonyl butoxide, organophosphorus compounds or carbamates may increase toxicity by mechanisms involving inhibition of microsomal oxidation (Anadon *et al.*, 2009).

These insecticides can also produce cardiotoxic, neurobehavioral, reproductive and developmental effects in animals and humans (Vijverberg and van den Bercken, 1990; Wolansky and Harril, 2007; Gupta, 2009; Malik *et al.*, 2011).

TREATMENT

There is no specific antidote for pyrethroid toxicity; animals should be treated symptomatically. The main treatment for dermal exposure is to wash the animal with a mild detergent and water. Do not use any shampoos that contain additional insecticides as this could increase exposure to insecticides. Large and small animals should be treated the same. The pyrethroids bound to the skin cannot be removed by washing with soap and water, but dermal paresthesia can be reduced by applying corn oil to the site(s) of application. For oral exposure, emetics or gastric lavage can be used to empty the stomach, if within 1–2 hours of ingestion. Activated charcoal and a saline or sorbitol cathartic will reduce oral absorption and increase elimination.

Supportive therapy using diazepam or barbiturates to control hyperexcitability or seizures can be used. Phenothiazine tranquilizers should not be used because



they can lower the threshold for seizures. Atropine can be used to control excess salivation or gastrointestinal hypermotility.

The prognosis for pyrethroid toxicity is usually good because of the low toxicity.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Pyrethroid insecticides, being of plant origin, are attractive to people that prefer to use organic insecticides on their companion animals or livestock, or who are engaged in organic food production. Pyrethrin insecticides, while toxic to selected species, have a wider margin of safety than organophosphate or organochlorine insecticides. Biomarkers of exposure to pyrethroids are being investigated. Urine is the matrix that is being most heavily investigated to determine which metabolites can be used to identify exposure to the parent compound. Advances in analytical methods to detect low concentrations of the pyrethroid metabolites will allow improved assessment of exposure in the future.

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# Neonicotinoids

Steve M. Ensley

## INTRODUCTION

Neonicotinoids are a new class of insecticides with widespread use in veterinary medicine and crop production. The neonicotinoid insecticides include imidacloprid, acetamiprid, dinotefuran, thiamethoxam and clothianidin. In the last decade neonicotinoid insecticides have been the fastest growing class of insecticides in modern crop protection because of the widespread use against a broad spectrum of sucking and certain chewing pests (Jeschke *et al.*, 2010). Neonicotinoids have a relatively low risk for nontarget organisms and the environment, a high target specificity to insecticide and versatility in application methods (Cresswell, 2011). The neonicotinoids have been implicated in the Colony Collapse disorder in bees but this has not been proven.

Imidacloprid is a neonicotinoid compound that is used as an insecticide for dermal application on animals, for termite and grub control and as an insecticide for crop protection. The neonicotinoids act on nicotinic receptors in insects and vertebrates. To reduce toxicity to mammals and increase toxicity to insects, neonicotinoid compounds have been selected that are highly specific for subtypes of nicotinic receptors that occur in insects. The neonicotinoids do not readily pass the blood–brain barrier, further reducing the potential for mammalian toxicity (Yamamoto *et al.*, 1995). When administered orally, imidacloprid is rapidly absorbed, metabolized primarily in the liver and excreted primarily in urine. Imidacloprid does not accumulate in the body, and it is not carcinogenic, mutagenic, teratogenic nor a reproductive toxicant. Imidacloprid has a high margin of safety due to the high insecticidal specificity and low mammalian toxicity (Nagata *et al.*, 1999).

## BACKGROUND

The neonicotinoids were developed in the late 1970s by chemists at Shell Chemical Company doing research with the heterocyclic nitromethylenes for use as insecticides (Soloway *et al.*, 1978; Schroeder and Flattum, 1984). The neonicotinoids and chloronicotinyls are a separate class of compounds from the nicotinoids (Tomizawa and Yamamoto, 1993). Chemists at Nihon Bayer Agrochem discovered imidacloprid in 1984 when the 3-pyridylmethyl group was added to the nitromethylene heterocyclic parent molecule. This addition to nitromethylene greatly increased the insecticidal activity and reduced mammalian toxicity. After imidacloprid was discovered several other analogs with the 6-chloro-3-pyridylmethyl moiety have been developed, such as acetamiprid, nitenpyram and thiacloprid (Takahashi *et al.*, 1992; Minamida *et al.*, 1993; Yamada *et al.*, 1999). Figure 48.1 shows the structure of imidacloprid.

## PHARMACOKINETICS/ TOXICOKINETICS

There are two routes of imidacloprid metabolism in mammals. The first route of metabolism involves oxidative cleavage of imidacloprid to imidazolidine and 6-chloronicotinic acid. The imidazolidine moiety is excreted in the urine. The 6-chloronicotinic acid is further degraded by glutathione conjugation to a derivative of mercapturic acid, then to methyl mercaptonicotinic acid. The mercaptonicotinic acid is then conjugated

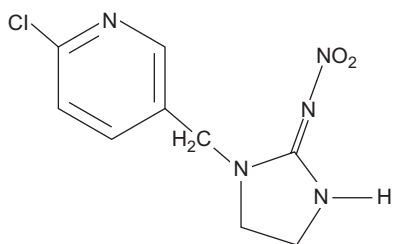


FIGURE 48.1 Structure of imidacloprid.

with glycine to form a hippuric acid conjugate that is excreted. A second route of metabolism involves hydroxylation of the imidazolidine ring followed by elimination of water and formation of an unsaturated metabolite. Specific information on the toxicokinetics of the rat can be found in [Thyssen and Machemer \(1999\)](#).

The following studies were conducted by Bayer CropScience ([Sheets, 2001](#)). Acute oral toxicity of imidacloprid in rats has been demonstrated at doses above 300 mg/kg with 100% mortality at 500 mg/kg. At doses in rats above 300 mg/kg, clinical signs were observed with 15 minutes of dosing and recovery was observed within 8 to 24 hours. A subchronic 13-week oral dosing study of imidacloprid in rats also demonstrated toxicity at a dose of 300 mg/kg in male rats. At a dose of 300 mg/kg in male rats, hypertrophy of hepatocytes and sporadic cell necrosis was observed in the liver. This mild liver damage was not observed after a 4-week recovery period. Serum alkaline phosphatase and alanine aminotransferase were elevated in male and female rats treated at 300 mg/kg and above. In dogs, a 13-week oral dose of imidacloprid at 15 mg/kg and above produced a tremor that increased with dose. A 52-week study in dogs at oral doses up to 72 mg/kg/day of imidacloprid did not produce tremors. The no observed effective level (NOEL) for this chronic oral exposure dog study was 15 mg/kg. Carcinogenicity was not observed in rats dosed orally at doses up to 103 mg/kg for 2 years. Studies confirm that imidacloprid is non-mutagenic, non-embryotoxic and non-teratogenic.

The most common adverse effects observed with dosing of neonicotinoids at low levels is decreased activity and tremors, impaired pupillary function (dilated or pinpoint pupils) and incoordinated gait; hypothermia is observed at higher doses. At lethal doses, deaths are observed within 4 hours of dosing. If death did not occur, recovery was observed within 8 to 24 hours of dosing.

## MECHANISM OF ACTION

Absorption and distribution of imidacloprid in rats occurs within 1 hour following oral administration. In rats more than 90% of a dose of imidacloprid is

eliminated within 24 hours. Approximately 80% of the dose is excreted by the urine with the remainder eliminated in the feces. Imidacloprid is not distributed to the central nervous system, fatty tissues or bone. This indicates that the blood-brain barrier allows little distribution to the central nervous system for imidacloprid in particular and the neonicotinoids in general.

The neonicotinoids act on post-synaptic nicotinic receptors ([Buckingham \*et al.\*, 1997](#)). These receptors are located entirely in the central nervous system of insects. Imidacloprid acts on at least three different subtypes of nicotinic receptors in the cockroach. Neonicotinoids cause a bi-phasic response: an initial increase in the frequency of spontaneous discharge followed by a complete block to nerve propagation. Insecticidal activity is increased by adding synergists that inhibit oxidative degradation ([Liu and Casida, 1993](#)).

Mammalian tissue also contains multiple subtypes of nicotinic receptors. The various subtypes are formed from different combinations of nine  $\alpha$ , four  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  subunits ([Tomizawa \*et al.\*, 1999](#)). Nicotinic receptors in mammals are located in the autonomic ganglia, skeletal muscle, spinal cord and in different regions of the brain. Neonicotinoids have much lower activity in vertebrates compared to insects due to the different binding properties of the various receptor subtypes ([Yamamoto \*et al.\*, 1998](#); [Tomizawa and Casida, 2011](#)). Acute toxicity of the neonicotinoids in mammals is related to the potency at the  $\alpha_7$  nicotinic receptor subtype with the activity at the  $\alpha_4$ ,  $\beta_2$ ,  $\alpha_3$  and  $\alpha_1$  receptors having a decreasing effect on toxicity. Toxicity in mammals involves complex interactions at multiple receptor sites with some of the receptor types even having a combination of agonist and antagonist effects on the synapse.

## TREATMENT

There is no specific antidote to treatment of overdoses of imidacloprid. Treatment is symptomatic. If the exposure is dermal, washing the affected animal with soap and water is indicated. If the exposure is oral, emetics, adsorbents or cathartics could be used depending on whether vomiting is occurring spontaneously or not. Absorption and elimination of imidacloprid is rapid so immediate supportive care should allow for rapid recovery.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

The neonicotinoids have a wide safety margin in mammals and the insecticidal activity can be targeted so this class of compounds can have wide future use as an insecticide.

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# Amitraz

Ramesh C. Gupta

## INTRODUCTION

Amitraz is a triazapentadiene compound, which is a member of the formamidine pesticide family. It has a chemical formula of  $C_{19}H_{23}N_3$  and molecular weight 293.41. Its chemical structure is shown in [Figure 49.1](#). Amitraz is commonly used in agriculture and horticulture as an insecticide and acaricide to control red spider mites, leaf miners, scale insects, aphids and other infestations. Amitraz is currently approved in the United States for various applications, including use on both food producing and companion animals. It is used to control ticks, mites, lice and many other pests on dogs, sheep, cattle and pigs. Amitraz is available as a wettable or pour-on powder, an emulsifiable liquid and a spray. It is also used as an active ingredient in formulations with other insecticides, such as metaflumizone (ProMeris®). Amitraz is sold under various product names, including Aazdieno, Acadrex, Acarac, Adrizan, Aludex, Amitik, Amitraze, Avartin, Baam, Bumetran, Ectodex, Edrizan, Kenaz, Mitac, Maitac, Metaban, Mitaban, Preventic, Ridd, Tickoff, Taktic, Triatix, Ovasyn, Ovidrex and many others. Of course, Mitaban, Preventic and Taktic are the three most popular brand names for amitraz. Amitraz poisoning is frequently encountered in dogs and cats ([Grossman et al., 1993](#); [Gunaratnam et al., 1993](#); [Hugnet et al., 1996](#); [Andrade et al., 2004](#)). In humans, poisoning occurs due to widespread use of amitraz veterinary products ([Ertekin et al., 2002](#); [Proudfoot, 2003](#); [Yilmaz and Yildizdas, 2003](#); [Aagin et al., 2004](#); [Avsarogullari et al., 2006](#)). Amitraz is not recommended for use in cats and horses as it causes toxicity. This chapter describes the toxicity of amitraz in animals with a special focus on dogs and cats.

## BACKGROUND

Amitraz is a broad spectrum insecticide and acaricide used in veterinary medicine and in agriculture and horticulture throughout the world since 1974. Amitraz has rapid action on the control of animal ectoparasites, such as mites, ticks, lice, etc., and it persists on hair and wool long enough to control all stages of the parasite. Amitraz was a restricted use pesticide in 1985 because some studies showed it caused cancer in mice. But re-evaluation of the evidence has led to the current classification of amitraz as an unrestricted or general use pesticide. In veterinary medicine, the most common use of amitraz is in tick collars, which contain 9% amitraz as an active ingredient. A collar for a large size dog contains 2.4g of amitraz ([Hugnet et al., 1996](#)). Amitraz is available as a 19.9% topical solution for dogs. All tickicide dips for cattle and sheep contain 0.025% amitraz as the active ingredient. In France, tick collars contain 8–9% amitraz, and external lotions contain 5–12.5% amitraz. The amitraz-containing product Metaban, which is a liquid concentrate and labeled for veterinary use, is commonly used for demodectic mange (*Demodex canis*) in dogs ([Farmer and Seawright, 1980](#)). Amitraz is also used to control ectoparasites on cattle, sheep, goats and pigs. Amitraz is not recommended for cats and horses ([Auer et al., 1984](#); [Gunaratnam et al., 1993](#)). There are several reports that have described amitraz poisoning in animals ([Roberts and Argenzio, 1979](#); [Turnbull, 1983](#); [Auer et al., 1984](#); [Hsu et al., 1984](#); [Grossman et al., 1993](#); [Hugnet et al., 1996](#); [Andrade et al., 2004](#)). With dogs, poisoning is most often associated with accidental ingestion of the collar, resulting in severe toxicity and sometimes fatal poisoning ([Grossman et al., 1993](#); [Hugnet et al.,](#)

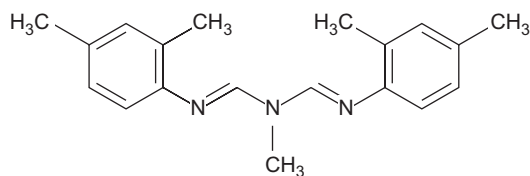


FIGURE 49.1 Chemical structure of amitraz.

1996). Amitraz should not be used in diabetic animals as it adversely affects the levels of glucose and insulin (Hsu and Schaffer, 1988).

## PHARMACOKINETICS/ TOXICOKINETICS

Amitraz is a highly lipid soluble compound that is rapidly absorbed following oral ingestion or dermal application, thus making exposure potentially dangerous for animals as well as humans. In the stomach, amitraz can be metabolized to as many as six metabolites and some of them are potentially toxic. Dogs receiving amitraz (100mg/kg, PO) in a gelatin capsule revealed the pharmacokinetic parameters as follows: area under the curve ( $AUC_{0-48h} = 265.3 \pm 12.3 \text{ mg/h/ml}$ ), elimination  $t_{1/2}$  ( $23.4 \pm 2.3 \text{ h}$ ), time to reach peak plasma concentration ( $t_{max} = 5.0 \pm 0.7 \text{ h}$ ), and mean peak plasma concentration ( $C_{max} = 20.7 \pm 2.3 \text{ mg/L}$ ) (Hugnet *et al.*, 1996). From these data it is clear that amitraz has a long elimination half-life and a significant amount is absorbed, which is accountable for most of the observed signs. There is a strong association between plasma amitraz concentrations and clinical manifestations in dogs (Hugnet *et al.*, 1996). Recently, Marafon *et al.* (2010) reported similar findings in cats experimentally intoxicated with 0.4% amitraz bath dip, which was eight times greater than the recommended concentration. Hugnet *et al.* (1996) observed that clinical signs of toxicosis usually appeared around 1h after ingestion, with plasma concentration around 5mg/L, and signs lasted until the concentration of amitraz decreased. This information can be used to monitor accidental ingestion of amitraz by dogs. In ponies and sheep, amitraz has a brief half-life after intravenous (IV) administration because it is hydrolyzed in the blood by formaminidases (Pass and Mogg, 1995).

The two major metabolites of amitraz are 2,4-dimethyl-formanilide and N-(2,4-dimethylphenyl)-N'-methylformamidine. The former metabolite is a relatively weak methemoglobin-former in dogs and man. These metabolites are further catabolized to 2,4-dimethylaniline and ultimately to 4-amino-3-methylbenzoic acid, which is the principal metabolite found in the urine and liver (Aronson *et al.*, 1988; Jones, 1990).

In essence, amitraz and its metabolites are excreted primarily in the urine and feces.

## MECHANISM OF ACTION

Amitraz kills mites, ticks and other parasites by interfering with their nervous system. The tick's sharp barbed mouth parts become paralyzed and cannot pierce the skin, thereby inhibiting the tick from feeding on dogs. Attached ticks will then detach.

Amitraz in higher doses has been shown to produce toxicity in several animal species by stimulating  $\alpha_2$ -adrenergic receptors ( $\alpha_2$ -AR) resulting in impairment of consciousness, respiratory depression, convulsions, bradycardia, hypotension, hypothermia and hyperglycemia. In early studies, amitraz produced effects similar to that produced by a pure  $\alpha_2$ -AR agonist drug such as clonidine (Costa *et al.*, 1988; Jorens *et al.*, 1997). In a recent experimental study, Marafon *et al.* (2010) observed significant declines in heart rate and respiration rate of cats intoxicated with amitraz. Electrocardiography (ECG) on an amitraz-poisoned English bulldog revealed prolonged QT intervals (Malmasi and Ghaffari, 2010). Amitraz acts centrally to influence blood pressure and heart rate by  $\alpha_2$ -AR agonism which causes a reduction in peripheral sympathetic tone (Kobinger, 1978; Cullen and Reynoldson, 1990). In the peripheral vasculature, both  $\alpha_1$  and  $\alpha_2$ -ARs are involved in contributing to the vasoconstrictor action of amitraz which results in hypotension. It is suggested that the central  $\alpha_2$ -AR agonist activity of amitraz is responsible for CNS depression (Cullen and Reynoldson, 1990).

In addition to being an  $\alpha_2$ -AR agonist, amitraz is a potent inhibitor of the enzyme monoamine oxidase (MAO), which is responsible for degrading the neurotransmitters norepinephrine and serotonin, resulting in behavioral and neurotoxic effects (Aziz and Knowles, 1973; Moser and Macphail, 1988). Amitraz is also known to cause inhibition of prostaglandins (Yim *et al.*, 1978).

## TOXICITY

### Acute toxicity

Acute toxicity data of amitraz is available for laboratory animals. Oral  $LD_{50}$  is 650mg/kg in rats and 1600mg/kg in mice. Values of dermal  $LD_{50}$  in rabbits and rats are >200mg/kg and 1600mg/kg, respectively. Oral  $LD_{50}$  values of amitraz for dog, pig, guinea pig and baboons are reported to be 100, 100, 400 and 100mg/kg, respectively. The inhalation  $LC_{50}$  (6h) of amitraz for rats is 65mg/L

of air. The oral LD<sub>50</sub> in birds (bobwhite) is estimated at 788mg/kg, and dietary LD<sub>50</sub> in mallard is 7000mg/kg and in Japanese quail is 1800mg/kg. LC<sub>50</sub> is 0.74ppm in rainbow trout, 0.5ppm in bluegill and 3.2–4.2ppm in harlequin fish. The EPA classifies amitraz as a Class III slightly toxic pesticide.

Signs of acute amitraz poisoning in male and female rats treated with 440mg/kg and 365mg/kg, respectively, include coolness to touch, reduced spontaneous activity, increased episodes of activity, such as aggression in response to handling, and signs of debilitation (Hayes and Laws, 1991). Due to its widespread use, acute amitraz poisoning is often encountered in dogs and cats. Poisoning may occur by oral, inhalation or dermal exposure, but poisoning is most often via the oral route. Onset of clinical signs is noted within 30min to 2h after ingestion. Clinical signs of poisoning include GI disturbance, nausea, vomiting, diarrhea, staggering, disorientation, CNS and respiratory depression, bradycardia, hypotension and hypothermia. Biochemical changes include hyperglycemia and elevation of liver enzyme (transaminases) activity. In dogs, following a topical application, amitraz has been shown to increase plasma glucose and decrease insulin release when dogs were dipped at twice the recommended concentration (Hsu and Schaffer, 1988). When amitraz is formulated in xylene and propylene oxide, signs such as depression, ataxia, stupor and coma are likely attributed to the xylene and propylene oxide (Jones, 1990). Generally, signs and symptoms of amitraz poisoning subside within 24–48h, while in some cases it may take 7–10 days.

In dogs and cats, common side effects of the pesticide include anorexia, sedation and a dry skin and hair coat. Serious side effects include low blood pressure, hypothermia, hyperglycemia, mydriasis, bradycardia, slow intestinal rate, incoordination, ataxia, vasoconstriction, vomiting, diarrhea, seizures and in rare instances death. Animals exposed to amitraz may show signs of CNS stimulation or CNS depression, depending on the dose level and to some extent the species involved. Generally, at low doses CNS stimulation may occur, as manifested by hyperactivity to external stimuli such as handling and considerably increased food consumption (Pfister and Yim, 1977). High doses of amitraz have a CNS depressive effect with reduced spontaneous activity, bradycardia, respiratory depression and hypothermia. Oglesby *et al.* (2006) observed renal cortical necrosis and hemorrhage in a dog, which died from acute renal failure following ingestion of an amitraz-formulated dip. Untreated dogs and cats usually go into a coma and die from respiratory failure. Surviving animals show complete recovery from all signs and symptoms in about 7–10 days, even when exposed to higher doses (Bonsall and Turnbull, 1983).

Evidence from animal studies suggests that amitraz is a neurotoxicant (Moser and Macphail, 1988). Amitraz has been shown to produce many behavioral and physiological changes in rats. Amitraz at a dose greater than 100mg/kg caused inhibition of monoamine oxidase (MOA) within 2h of dosing and lasted up to 7 days. Amitraz appears to be more selective for type B-MAO when given *in vivo*, although MAO-A was also inhibited at doses  $\geq 300$ mg/kg. However, no selectivity was indicated by the IC<sub>50</sub> values determined *in vitro* (IC<sub>50</sub> = 31 and 28 $\mu$ M for MAO-A and MAO-B, respectively). Findings revealed that the MAO inhibition is probably not due to amitraz-induced alterations in motor activity. Amitraz produced only negligible inhibition of acetylcholinesterase at very high doses. In a dose-dependent manner, amitraz produced depressed arousal and rearing activity, hypothermia, body weight loss; and autonomic changes including ptosis, mydriasis, chromodacryorrhea resulting in facial crustiness, loss of the pupil reflex and decreased defecation in rats (Moser, 1991).

Horses poisoned by amitraz may show clinical signs of tranquilization, depression, ataxia, muscular incoordination and impaction colic that can last up to a week, followed by mild dehydration and acidosis.

## Chronic toxicity

In chronic 2-year feeding trials, rats receiving 50mg/kg/day in their diet or dogs receiving 0.25mg/kg/day of amitraz did not show any ill effects. Dogs given 1mg/kg/day exhibited signs of slight CNS depression and hypothermia, and blood analysis revealed significant hyperglycemia.

Studies suggest that amitraz exerts endocrine disrupting activity and reproductive and developmental toxicity. Male and female rats receiving amitraz at 200mg/kg/day for 10 weeks showed decreased fertility. Female mice treated with amitraz orally (50mg/kg/day) for 5 days and then mated showed a slight increase in loss of fetuses and a decrease in the number of living offspring. When male mice were given the same dose for the same duration and then mated, the resulting embryos were significantly less likely to grow in the mother's uterus. Rabbits who received 25mg/kg/day of amitraz from days 6 to 18 of pregnancy had fewer and smaller litters (Meister, 1994).

Being an  $\alpha_2$ -AR agonist, amitraz is reported to adversely affect the mammalian reproductive system by binding to presynaptic  $\alpha_2$ -AR in the hypothalamus, thus inhibiting noradrenalin release and decreasing gonadotropin-releasing hormone secretion (Altobelli *et al.*, 2001). In addition, several *in vivo* and *in vitro* studies have demonstrated that amitraz is a reproductive toxicant (Goldman and Cooper, 1993; Young *et al.*, 2005).

Amitraz also has the potential for teratogenic activity. In rats treated with 12 mg/kg/day of amitraz from day 8 to 20 of pregnancy, the offspring were heavier but had less bone development than the offspring of untreated rats (Hayes and Laws, 1991). In a recent investigation, Kim *et al.* (2007) showed that amitraz administered during the entire pregnancy period in rats is embryotoxic and teratogenic at the maternally toxic dose (i.e., 30 mg/kg/day) and is minimally embryotoxic at a minimal maternally toxic dose (i.e., 10 mg/kg/day). Amitraz and its metabolite 2,4-dimethylaniline have also been shown to induce teratogenic effects in frog embryos (Osano *et al.*, 2002).

Studies in laboratory animals have shown that amitraz has no potential for mutagenic activity and does not cause damage to DNA (Hayes and Laws, 1991). Amitraz can cause tumors in female mice but not in male mice or male or female rats. EPA has established the teratogenic no observed effect level (NOEL) in rats at 12 mg/kg/day (Walker and Keith, 1992). The teratogenic NOEL in rabbits is 25 mg/kg/day.

## Diagnosis

Most of the time, poisoning in animals (especially dogs and cats) with amitraz is acute. Diagnosis is based on history of exposure, presence of the collar's pieces in the stomach on X-ray, clinical signs of toxicity associated with stimulation of  $\alpha_2$ -AR, and residue analysis. Due to a long half-life, residue of amitraz and its major metabolites can be detected in the plasma of a poisoned animal using GC coupled with a nitrogen-phosphorus detector or a thermionic-specific detector (Ameno *et al.*, 1991; Marafon *et al.*, 2010), or HPTLC coupled with a UV detector (Hugnet *et al.*, 1996).

## TREATMENT

In most cases, symptomatic and supportive therapies are of great value. Remove the tick collar or any other source of amitraz from pets. IV fluids are recommended. In the case of dermal exposure, bathing is helpful. For oral ingestion, induction of vomiting and gastric lavage is recommended. Administration of activated charcoal with a saline cathartic is also beneficial. Animals should be kept in the warm, as amitraz causes hypothermia. Continuous monitoring of cardiovascular, respiratory and central nervous systems, and of glucose and insulin levels is advised.

Studies from animals suggest that the  $\alpha_2$ -AR antagonists (yohimbine and atipamezole) can reverse amitraz-induced toxicity. In dogs, yohimbine has been found to

be very effective. Bradycardia and hypotension respond to yohimbine administration (Hsu and Schaffer, 1988). Dogs severely poisoned with amitraz have been successfully treated with low doses (50  $\mu$ g/kg, IM) of the potent  $\alpha_2$ -AR antagonist atipamezole (Hugnet *et al.*, 1996). Doses of atipamezole can be repeated after 3 to 4 h, if necessary. Atipamezole can control all the clinical effects of amitraz within 20 min. With good care and aggressive treatment, even severe cases can be recovered.

## CONCLUSIONS

Amitraz is widely used as an insecticide and acaricide in veterinary medicine. Dogs and cats are intoxicated due to accidental ingestion of amitraz-containing tick collars and other products. Major toxic signs are associated with  $\alpha_2$ -adrenergic receptor ( $\alpha_2$ -AR) agonism, such as CNS depression, hypotension, bradycardia, and hypothermia. Increased blood glucose and decreased insulin secretion are characteristics of amitraz poisoning and are also due to  $\alpha_2$ -AR stimulation. With timely administration of an  $\alpha_2$ -AR blocker (yohimbine or atipamezole), and conventional therapeutic measures, even seriously intoxicated cases can recover.

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# Fipronil

Arturo Anadon and Ramesh C. Gupta

## INTRODUCTION

Fipronil is a member of a new class of insecticides called phenylpyrazoles. Chemically, it is a (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole). The chemical structure of fipronil is shown in Figure 50.1. Fipronil is an active ingredient of one of the popular ectoparasiticides products Frontline™. There are currently two forms of Frontline preparation commercially available (the spray and the spot-on topical application) for dogs and cats. The product is meant to kill fleas and all stages of brown dog ticks, American dog ticks, lone star ticks, which may carry Lyme disease and mites (Cutler, 1998; Hutchinson *et al.*, 1998; Anonymous, 2000). Fipronil is used in combination with methoprene (9.8% fipronil/11.8% methoprene for cats; 9.8% fipronil/8.8% methoprene for dogs) for additional control of immature flea stages. Fipronil is also formulated as insect bait for roaches, ants and termites; sprays for pets; and as a granular turf product to control mole crickets. Presently, fipronil is widely used in agriculture for soil treatment and seed coating.

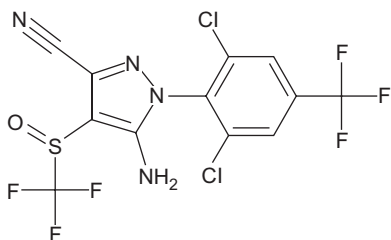


FIGURE 50.1 Chemical structure of fipronil.

Currently, fipronil-based products with various trade names (Chipco®, Choice, ICON 6.2FS™, and Over n' Out™, TeckPac, Frontline) have gained popularity worldwide for pest management, including residential insect control, rice and cotton production, and turf-grass management. EPA has determined fipronil to be safe for use on dogs and cats, with no harm to humans who handle these animals. Poisoning cases from accidental or misuse of fipronil may occur in animals. This chapter describes the toxicity of fipronil in various species of animals, birds and fish.

## BACKGROUND

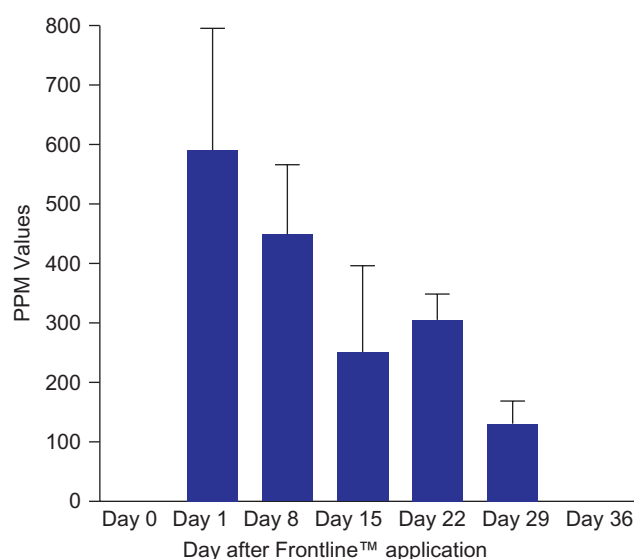
Fipronil was first discovered by Rhone-Poulenc Agro in 1987 and is used worldwide in agriculture. It represents the second generation of insecticides that acts through a different mechanism compared to other conventional insecticides. It is poorly soluble in water, delivered in very small amounts and does not leach into the groundwater. In 1996, it was registered as a pesticide in the U.S. (Bobe *et al.*, 1998). It can be formulated as roach or ant baits, flea and tick sprays for pets, and in granular turf products to control mole crickets (Kidd and James, 1991; EPA, 1996). In addition, fipronil is used for soil treatment and on crops to protect from insects. It is currently used in over 50 countries and has good adulticide activity against fleas and ticks. It has significant residual activity and for small animal use is available as a spray or spot-on product. Sunlight, immersion in water and bathing do not significantly impact the performance of

this compound and it kills adult fleas within 24 hours (Kunkle, 1997). Fipronil has also been used to treat some mite infections.

Most of the time poisoning cases of fipronil occur in dogs and cats due to accidental ingestion or licking the product Frontline. Frontline preparation for dogs contains 132mg fipronil in a volume of 1.34ml tube that is meant for a topical application. In humans, poisoning is mainly due to accident or suicidal attempt. In a recent report, the Paris Poison Center, France, recorded 81 human cases of fipronil exposure from 1994 to 1999. Out of these 81 cases, 57 involved veterinary ectoparasitocides and seven used domestic insecticide preparations (Gasmi *et al.*, 2001).

## PHARMACOKINETICS/ TOXICOKINETICS

Fipronil in Frontline preparation (132 mg in a 1.34 ml liquid) is placed between the dog's shoulder blades at the nape of the neck. After application, fipronil spreads and sequesters in the lipids of the skin and hair follicles, and continues to be released onto the skin and coat, resulting in long-lasting residual activity against fleas and ticks. Residue of fipronil lasts on a dog's hair coat for about a month. The maximum concentration of fipronil on the canine hair coat is found 24h after a single application of Frontline top spot (Jennings *et al.*, 2002). With a descending concentration trend fipronil residue can be detected on dog's hair coat for a period of 30 days (Figure 50.2). Although fipronil binds to the lipids of the skin cells and



**FIGURE 50.2** Transferable residue of fipronil from the dog coat after a single application of Frontline spot-on.

hair follicles, the transferable residue can be detected up to a month after application. Studies have revealed that in rats, fipronil is excreted mainly in the feces (45–75%) and little in the urine (5–25%). In rats, once absorbed, fipronil is rapidly metabolized, and then the biotransformation products are widely distributed in tissues. Significant amounts of metabolites can persist in the tissues, particularly in fat and fatty tissues, for one week after treatment. The metabolite concentrations in fat and other tissues are greater with repeated low doses or a single high dose than with a single low dose. The long half-life (150–245 h in some cases) of fipronil in blood may reflect slow release of metabolites from fat. The compounds identified in feces and urine are the parent compound and the sulfone, the amide derived from the nitrile group and a cleavage product of the sulfone and its derivatives formed by further metabolism. The sulfone is the major metabolite in fat and tissues.

In another pharmacokinetics study the rats received either a single oral dose of labeled compound ( $^{14}\text{C}$ -fipronil-desulfinyl) at 1 or 10 mg/kg body weight or daily oral doses of unlabeled compound at 1 mg/kg body weight/day for 14 days, followed by a single oral labeled dose. In animals of both sex, elimination of the radiolabeled fipronil was much greater in the feces (46–70% of the dose) than in the urine with all dosing regimens. Appreciable metabolic products were found in the tissues 1 week after treatment, the highest concentrations being present in the fat and fatty tissues. The long half-life in blood was 183–195 h and fat:plasma ratio of the radiolabel compound was increased. Numerous metabolites or conjugates of fipronil desulfinyl were present in the urine and feces. Only unchanged fipronil desulfinyl was identified in the liver, fat, skin and body. There was no appreciable difference between male and female rats in the absorption, distribution, metabolism or excretion of fipronil after oral administration. Fipronil is degraded by sunlight to produce a variety of metabolites, one of which is fipronil desulfinyl. This metabolite is extremely stable, bioaccumulates in the fatty tissues and is more toxic than the parent compound in insects (EPA, 1998). Information on other metabolites of fipronil in the living system and nonliving system can be found elsewhere (Feung and Yenne, 1997; Aajoud *et al.*, 2003). This photoproduct exerts high neurotoxicity by blocking the GABA-regulated chloride channels. Fipronil has a greater specificity for the GABA receptor found in insects than mammalian GABA receptors. Fortunately, this metabolite is not formed in mammals. However, it does have high affinity towards the insect's GABA system, thereby contributing to fipronil's selective toxicity toward insects.

It is known that fipronil is an effective inducer of CYP isoforms in human hepatocytes. Fipronil can inhibit testosterone metabolism by CYP3A4. Since fipronil is a

hepatotoxicant at doses somewhat above those required for induction, the decrease may represent one of the initial toxic effects, with CYP3A4 induction being more sensitive than CYP1A1 induction. Fipronil has the potential to interact with a wide range of xenobiotics or endogenous chemicals that are CYP3A4 substrates. In the case of possible metabolic interactions based on the induction or inhibition of xenobiotic metabolizing enzymes, the concentrations of fipronil at the active site(s) become crucial.

## MECHANISM OF ACTION

In mammalian systems, the mechanism of fipronil is quite different from other classes of insecticides, and it is better understood in insects than in mammals. Fipronil sulfone is a major metabolite of fipronil in mammals and insects. In invertebrates (insects), fipronil or its metabolite non-competitively inhibits  $\gamma$ -aminobutyric acid (GABA)-induced ion influx by targeting the GABA-regulated chloride channels (Cole *et al.*, 1993). Consequently, fipronil binding blocks the inhibitory action of GABA, leading to neural hyperexcitation, and at sufficient concentrations, paralysis and death (Bobe *et al.*, 1998). Fipronil exhibits >500-fold selective toxicity to insects over mammals, primarily because of affinity differences in receptor binding between insect and mammalian receptors (Cole *et al.*, 1993; Grant *et al.*, 1998; Hainzl *et al.*, 1998; Kamijima and Casida, 2000; Ratra *et al.*, 2001; Zhao *et al.*, 2005; Narahashi *et al.*, 2010). In essence, fipronil binds more tightly to GABA<sub>A</sub> receptors in insects than in mammals. It is important to note that since fipronil sulfone is rapidly formed *in vivo*, the toxicological effects are likely due to the sulfone metabolite (Zhao *et al.*, 2005).

The toxicity of fipronil desulfinyl is qualitatively similar to that of fipronil, but the dose–effect curve for neurotoxic effects appears to be steeper for fipronil desulfinyl than for fipronil. Also, fipronil desulfinyl appears to have a much greater tendency than fipronil to bind to sites in the chloride ion channel of the rat brain GABA receptor. This finding appears to be consistent with the greater toxicity, relative to fipronil, of fipronil desulfinyl in the CNS of mammals.

Different studies have shown that fipronil reveals thyroid disrupting properties in rats. Mechanistic investigations conducted with fipronil in rats suggest that it does not interfere with the incorporation of iodine into thyroxine (T<sub>4</sub>), but it interferes with the biliary clearance of this hormone. This may trigger an increase in the concentration of thyroid-stimulating hormone by interference with the feedback mechanism and decreased thyroxine concentrations.

## TOXICITY

Fipronil produces toxicity in insects and mammals by the same mechanism; however, due to selective action, the toxicity is much more severe in insects than in mammals. Fipronil and other phenylpyrazole compounds exert neurotoxicity by blocking transmission of signals by inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA). These compounds bind within the chloride channels and consequently inhibits the flux of chloride ions into the nerve cell, resulting in hyperexcitation.

There are numerous reports regarding the effects of fipronil in small animals, birds and fish (<http://www.cdpr.ca.gov/docs/empmpubs/fatememo/fipronil.pdf>).

### Laboratory animals

Adequate acute toxicity data are available from small animals. Fipronil is moderately hazardous to rats (LD<sub>50</sub> = 97mg/kg body weight) and mice (LD<sub>50</sub> = 95mg/kg body weight). In rats, signs of toxicity and death were delayed for up to 4 days after a single dose or repeated oral doses of 75mg/kg body weight per day for up to 5 days.

Fipronil has moderate inhalation toxicity with an acute LC<sub>50</sub> of 0.682mg/L in rats (EPA, 1996).

It is nontoxic to slightly toxic via the dermal route, with a reported dermal LD<sub>50</sub> of greater than 2000mg/kg in rats (EPA, 1996). In rabbits, fipronil was found to be moderately hazardous after dermal application (LD<sub>50</sub> = 354mg/kg body weight; WHO, 1998–1999). In general, dermal absorption of fipronil is less than 1% after 24 h and therefore dermal toxicity is considered low. In a dermal toxicity study, fipronil was applied (0.5% in carboxymethylcellulose) to the intact skin of rabbits for 6 h per day, 5 days a week, for 3 weeks at doses of 0, 0.5, 1, 5 or 10mg/kg body weight per day. No dermal irritation was observed. At 10mg/kg body weight per day, body weight gains and food consumption were reduced in animals of either sex. Some animals showed hyperactivity. The NOAEL was identified to be 5mg/kg body weight per day. Primary dermal irritation in rabbits was examined in two studies. Fipronil was slightly irritating when moistened with corn oil before application, but was not irritating when moistened with water. Fipronil was slightly irritating in two studies of primary ocular irritation in rabbits. It did not sensitize the skin of guinea pigs when tested by the Buehler method but was a weak sensitizer in guinea pigs tested by the Magnusson-Kligman method (WHO, 1998–1999). This product was reported to be contraindicated in rabbits because deaths occurred when spray formulation of fipronil was used. Extra-label use in rabbits could



produce anorexia, lethargy, convulsions and death. In rabbits, fipronil produces serious adverse reactions, suggesting that this species is unusually sensitive to fipronil. Young rabbits are especially more sensitive than adults. Frontline has been considered to be improper for rabbits.

One reproductive toxicity study was performed in rabbits (WHO, 1998–1999). Rabbits treated with fipronil by gavage at doses of 0, 0.1, 0.2, 0.5 or 1 mg/kg body weight per day during gestation days 6–19 showed no developmental toxicity, but there were some signs of maternal toxicity (decreased body weight gain, decreased food consumption and reduced efficiency of food use at all doses). The NOAEL for developmental toxicity was identified to be 1 mg/kg body weight per day. Fipronil has also been found to be a developmental neurotoxicant in the PC12 cells and zebrafish models (Stehr *et al.*, 2006; Lassiter *et al.*, 2009).

### Dogs and cats

Several subacute and chronic toxicity studies of fipronil have been performed in dogs (WHO, 1998–1999). In a subacute toxicity study, fipronil was administered in gelatin capsules to dogs for 13 weeks at doses of 0, 0.5, 2 or 10 mg/kg body weight per day. Inappetence and decreased body weight gain and food consumption were noted in females at 2 and 10 mg/kg body weight per day. The NOAEL was reported to be 0.5 mg/kg body weight per day.

In a chronic study, fipronil was administered to dogs in gelatin capsules for 1 year at doses of 0, 0.2, 2 or 5 mg/kg body weight per day. At 2 mg/kg body weight per day and above, there were clinical signs of neurotoxicity (convulsions, twitching, tremors, ataxia, unsteady gait, rigidity of limbs, nervous behavior, hyper- or hypoactivity, vocalization, nodding, aggression, resistance to dosing, inappetence and abnormal neurological responses) in animals of both sex. One animal at 2 mg/kg body weight per day was killed because of poor condition related to treatment. The NOAEL was identified to be 0.2 mg/kg body weight per day. In another study, fipronil was administered in the diet at doses of 0, 0.075, 0.3, 1 or 3 mg/kg body weight per day for 1 year. At 1 mg/kg body weight per day, clinical signs of neurotoxicity (whole body twitching and extensor rigidity of limbs) were noted in females. There were no effects on triiodothyronine or thyroxine levels. The NOAEL was identified to be 0.3 mg/kg body weight per day.

A neurotoxicity study was performed in female dogs (WHO, 1998–1999). In this study, dogs received fipronil in capsules at doses of 0 (one animal) or 20 mg/kg body weight per day (four animals) until the appearance of neurotoxic signs in each animal, after which they were allowed to recover for 28 days. Severe neurotoxic signs

were observed at a dose of 20 mg/kg body weight per day during the treatment phase. Most animals appeared to recover, although one had exaggerated reflex responses and was excitable at the end of the recovery period.

Poisoning cases of fipronil occur in dogs and cats due to accidental ingestion/licking of Frontline product. There is some indication that dogs are more severely affected than cats. Application of Frontline spot-on to dogs and cats can cause skin irritation and/or hair loss at the site of application. Dermal hypersensitivity reactions may also occur. Common clinical signs of fipronil toxicosis are of CNS hyperexcitability, including tremors, convulsions, seizures and death (Grant *et al.*, 1998; Hainzl *et al.*, 1998; Kamijima and Casida, 2000).

### Birds and fish

On an acute and subchronic level fipronil is practically nontoxic to slightly toxic in waterfowl with an acute oral LC<sub>50</sub> of >2000 mg/kg and a 5-day dietary LC<sub>50</sub> of >5000 mg/kg in mallard ducks. The oral LC<sub>50</sub> for bobwhite quail is 11.3 mg/kg, and the LC<sub>50</sub> for 5-day dietary is 49 mg/kg (EPA, 1996). The sulfone metabolite is more toxic than the parent compound to certain bird species. This metabolite has shown a very high toxicity in game birds and moderate toxicity in waterfowl (EPA, 1996; Bobe *et al.*, 1997). Fipronil is highly toxic to rainbow trout and very highly toxic to bluegill sunfish with an LC<sub>50</sub> of 0.246 mg/L and 0.083 mg/L, respectively. WHO classifies fipronil as a Class II moderately hazardous pesticide.

## DIAGNOSIS

Diagnosis of a fipronil poisoning can be based on circumstantial evidence, clinical manifestations and chemical confirmation. Fipronil residue can be detected in the blood, tissue or hair, using GC/MS. Transferable residue of fipronil can be detected on a dog hair coat up to 4 weeks after a single spot-on application (Figure 50.2).

## TREATMENT

There is no specific treatment for the toxicity of fipronil, and therefore treatment relies upon symptomatic and supportive measures. If a dog or cat shows adverse reaction to topical application of Frontline, decontamination by washing at the site of application is advisable. The ingestion of any fipronil topical veterinary product

should be treated by the ingestion of water or milk. The most recommended methods of internal decontamination include gastric lavage, and administration of activated charcoal (1–4 g/kg) and cathartic agent within 3–4 hours.

If hypersensitivity skin reactions occur after application of any topical product, the animals should be submitted for a bath with a non-insecticidal shampoo and symptomatic treatment (e.g., antihistamines, hydrocortisone or antibiotics).

## CONCLUSIONS

Fipronil elicits neurotoxicity in mammals by inhibition of GABA<sub>A</sub>-gated chloride channels, producing hyperexcitability of CNS. Overdosage due to accidental ingestion often leads to serious toxicosis in dogs and cats. There is no specific antidote for the toxicity of fipronil. The manufacturer warns that the product may be harmful to debilitated, aged, pregnant or nursing animals and also stated that fipronil must not be used in kittens less than 12 weeks of age and on puppies less than 10 weeks old.

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# Macrocyclic lactone endectocides

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## INTRODUCTION

Macrocyclic lactones (MLs) consist of a number of compounds (doramectin, eprinomectin, ivermectin, milbemycin, moxidectin and selamectin) that are commonly used as insecticides, acaricides and nematicides in various animal species. Chemical structures of these MLs are shown in Figures 51.1–51.6. Ivermectin, a semi-synthetic ML, was the first to be obtained from *Streptomyces avermitilis*. Ivermectin/abamectin is a mixture of two homologs, i.e., ivermectin B<sub>1a</sub> and ivermectin B<sub>1b</sub> (Figure 51.1). It was introduced to the market in 1981 as a potent antiparasitic animal health drug. It has been found to be effective against virtually all external and internal parasites thus far tested, with the exception of trematodes and cestodes. The drug is approved at a very low dosage for the control of parasites in many animal species (cattle, sheep, swine, horse, cat and dog), but not approved in lactating cows, sheep and goats. In agriculture, ivermectin is used for its miticidal, insecticidal and acaricidal activities.

Although ivermectin poisoning has been reported in many animal species due to inadvertent or misuse of the product (Godber *et al.*, 1995; Haldrick *et al.*, 1995), a major concern of ivermectin toxicosis is in individuals of certain dog breeds, such as collies (Paul *et al.*, 1987), which are very sensitive to this drug due to a genetic defect in the blood–brain barrier (BBB). To date, over 11 dog breeds (Houston, *et al.*, 1987; Geyer *et al.*, 2007; Mosher and Court, 2010), Murray Grey cattle (Seaman *et al.*, 1987) and young animals have been reported to be more sensitive to ivermectin toxicosis.

Selamectin, a novel semi-synthetic avermectin, is marketed as Revolution™ (Pfizer). Revolution™ is a topical antiparasitic preparation recommended for use

in dogs and cats 6 weeks of age and older. Selamectin is used to kill fleas (*Ctenocephalides felis*) and ear mites (*Otodectes cynotis*) in dogs and cats. It is also indicated for the treatment and control of sarcoptic mange (*Sarcoptes scabiei*) and for the control of tick (*Dermacentor variabilis*) infestations in puppies, as well as for the treatment of hookworm (*Ancylostoma tubaeforme*) and roundworm (*Toxocara cati*) infections in kittens. Selamectin is also used to prevent heartworm disease caused by *Dirofilaria immitis*. Most recently, Jacsó *et al.* (2010) reported the efficacy of selamectin in the treatment of subcutaneous dirofilariosis in dogs caused by *Dirofilaria repens*. In addition to ivermectin and selamectin, there are many other macrocyclic lactones which pose a serious threat to animal health following accidental or overdose exposure. This chapter describes the toxicity of macrocyclic lactone endectocides in animals.

## BACKGROUND

Currently, there are many macrocyclic lactones (MLs) available in the market for their wide applications as insecticides, nematicides and acaricides. But ivermectin and its derivatives are most popular because of their efficacy and wide margin of safety. Ivermectin/abamectin is a mixture of two homologs, i.e., not less than 80% of 22,23-dihydroavermectin B<sub>1a</sub> and not more than 20% of 22,23-dihydroavermectin B<sub>1b</sub>. B<sub>1a</sub> and B<sub>1b</sub> components differ from each other by a single methylene group, have very similar biological and toxicological properties, and can, for all practical purposes, be considered equipotent (Campbell, 1989). The mixture is sold commercially

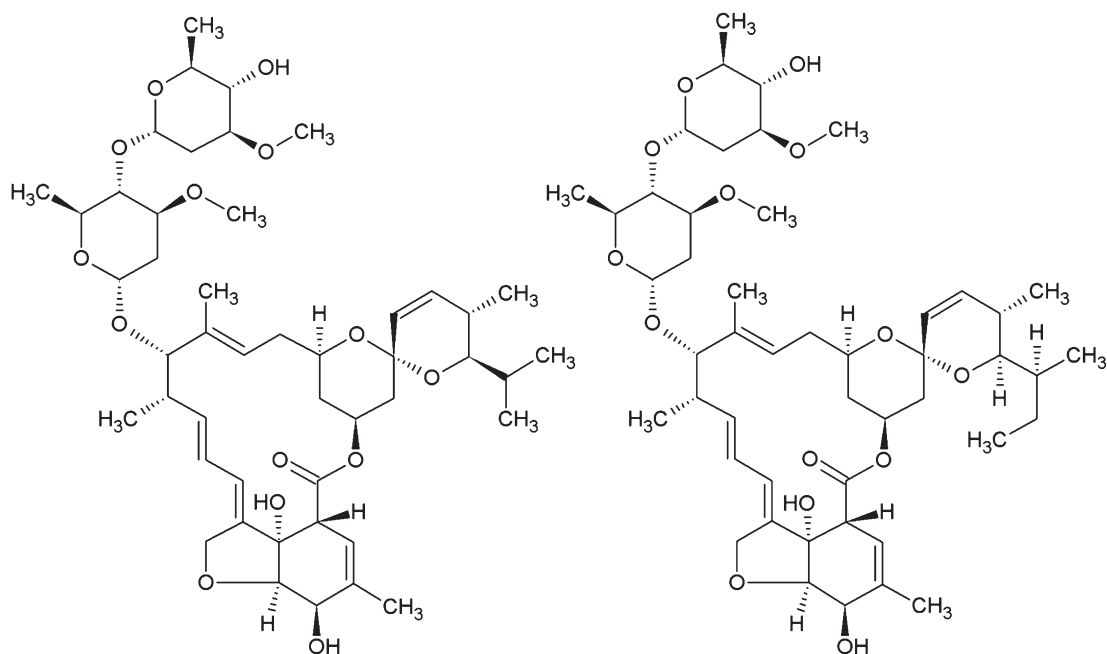


FIGURE 51.1 Chemical structure of abamectin (right, ivermectin B<sub>1a</sub>; and left, ivermectin B<sub>1b</sub>).

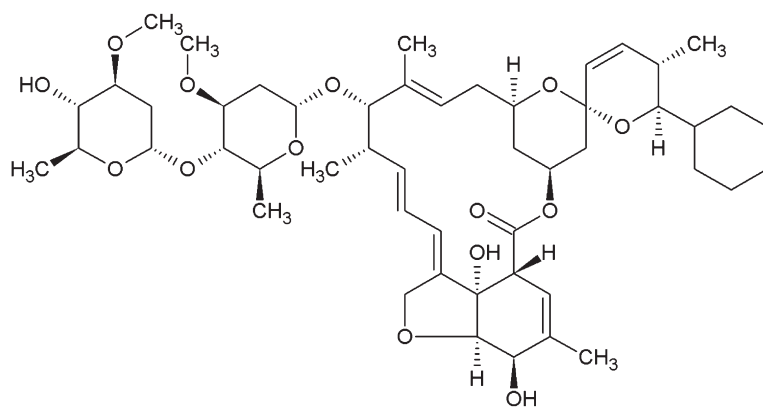


FIGURE 51.2 Chemical structure of doramectin.

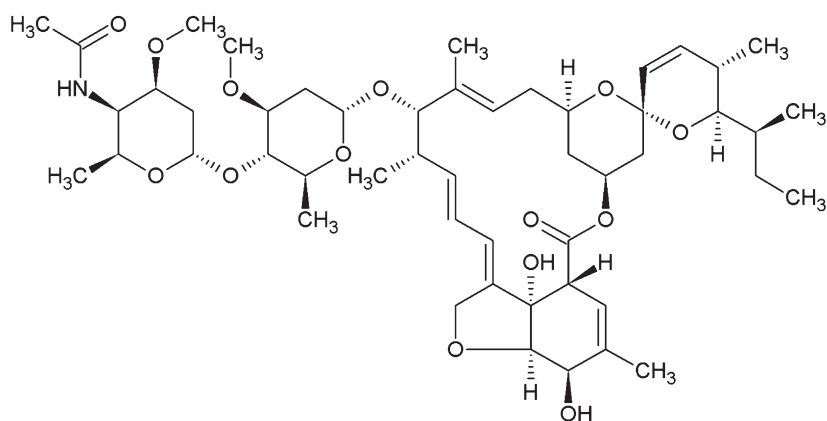


FIGURE 51.3 Chemical structure of eprinomectin.



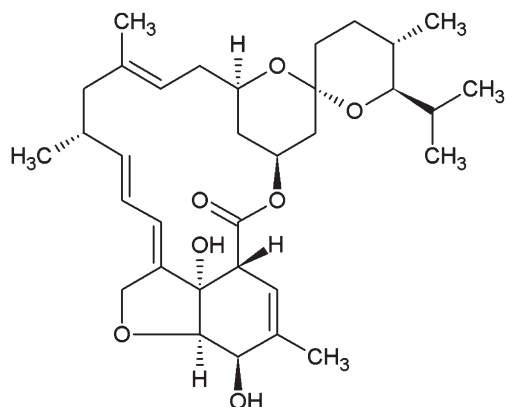


FIGURE 51.4 Chemical structure of milbemycin.

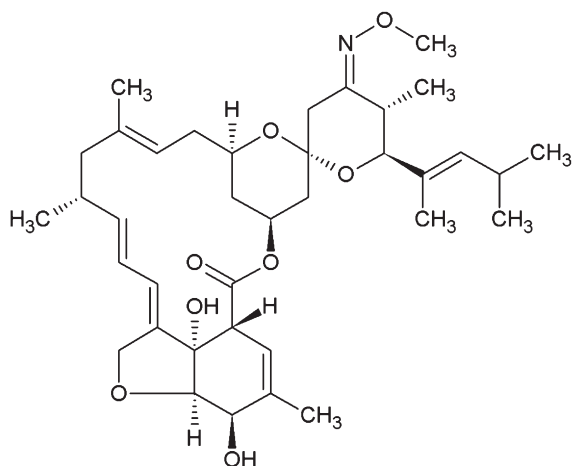


FIGURE 51.5 Chemical structure of moxidectin.

as abamectin. It is a drug of choice because of its broad spectrum nematicidal, insecticidal and acaricidal properties, and it has been approved for prophylactic use against heartworm (*Dirofilaria immitis*) infection in dogs.

Ivermectin is utilized at a very low dosage. It is effective against a wide range of helminths in sheep and cattle in a single oral or parenteral dose of 0.1 mg/kg body weight (Egerton *et al.*, 1979). It is recommended in a variety of species, including dogs, cats, sheep, goats, swine, feedlot beef cattle, horses and reindeer. The tolerances for beef cattle, reindeer, swine and sheep are 15, 15, 20 and 30 ppb ivermectin B<sub>1a</sub>, respectively (CFR, 1990). Ivermectin is not approved for lactating animals.

In the case of beagles the dose required for prevention of heartworm disease is 6 µg ivermectin/kg body weight, while the oral LD<sub>50</sub> of this drug in the same breed is 80,000 µg/kg body weight (Pullium *et al.*, 1985). However, certain dogs exhibit signs of toxicosis when given ivermectin at oral doses in the range of 100–2500 µg/kg body weight (Easby, 1984; Houston, 1987). Although this dosage level exceeds the approved heartworm preventive

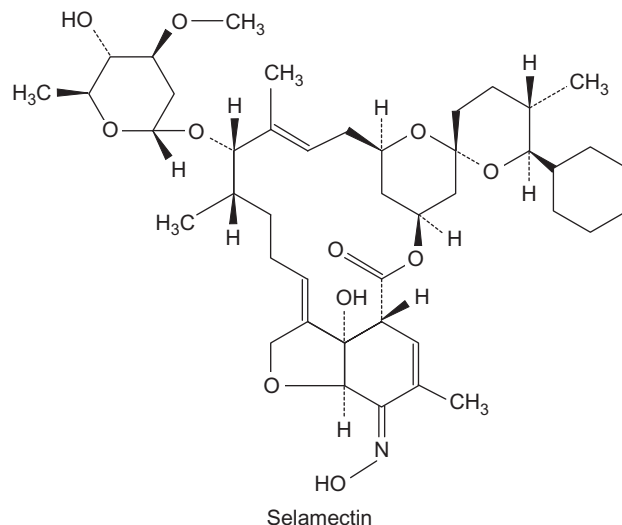


FIGURE 51.6 Chemical structure of selamectin.

dose for dogs, it has been administered inadvertently by the improper use of formulations which were meant for cattle, sheep, horses and swine. Additionally, oral dosages of ivermectin of 200–600 µg/kg are frequently used by veterinarians to manage Sarcptic and Demodectic mange in dogs. Poisoning in several animal species has been reported due to overdosage of ivermectin. Young animals in general are more sensitive than adults because their immature BBB are more permeable to ivermectin (Sanford *et al.*, 1988).

Topical application of Revolution™ (6 mg selamectin/kg body weight) has been shown to have a broad range of efficacy against many external and internal parasites of dogs and cats (Jacobs, 2000; Dryden *et al.*, 2001). Following topical administration of Revolution™ to a single site on the skin, selamectin is absorbed by the animal, enters the bloodstream and the GI tract, and it is ingested by the external and internal parasites as they feed on the treated host. Selamectin has been shown to have a direct parasitocidal effect following ingestion (Bishop *et al.*, 2000; Pfizer, 2001). Selamectin has a wide margin of safety, but poisoning can occur due to overdosage or accidental exposure.

Other MLs are variously used as injectable, oral and topical products in livestock, including abamectin (Duotin®, injectable), doramectin (Dectomax®, injectable and pour-on), eprinomectin (Eprinex®, pour-on) and moxidectin (Cydectin®; oral, injectable and pour-on) in dosages ranging from 200 to 1000 µg/kg. Moxidectin is also utilized as heartworm preventives in dogs at dosages of 3 µg/kg orally and 17 µg/kg as subcutaneous sustained-release injection (ProHeart®6). Milbemycin (Interceptor®) is used as an oral heartworm preventive in dogs at dosages of 500 µg/kg and cats at dosages of 2000 µg/kg.

## PHARMACOKINETICS/ TOXICOKINETICS

The extended antiparasitic activity of MLs in mammals is due to the unique pharmacokinetic profile of this class of compounds. MLs tend to have prolonged tissue and plasma residence times due to their relatively slow absorption, wide tissue distribution, low rate of metabolism and slow rate of elimination (Lanusse *et al.*, 2009). Characteristics of MLs that influence these kinetic properties include strong adsorption to gastrointestinal particulate digesta, extensive and reversible plasma-tissue exchanges, relatively high degree of lipophilicity, extensive biliary and p-glycoprotein mediated intestinal secretion and extensive enterohepatic recycling. With most MLs, the pharmacokinetic properties are dose dependent and highly dependent upon the formulation of the individual compounds. For instance, when injected subcutaneously, ivermectin formulations that are oil-based have a slower absorption than those based in glycerol or propylene glycol (Gonzalez *et al.*, 2007).

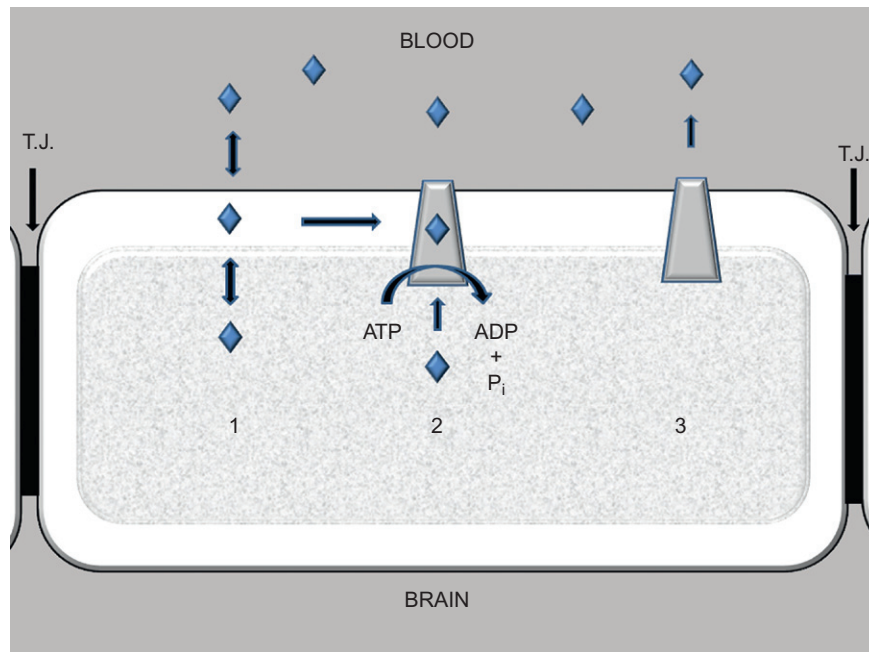
In general, MLs are well absorbed following injection, oral or topical administration, although species differences in absorption and bioavailability have been noted for some MLs. For instance, the bioavailability of selamectin following topical administration was 4.4% in dogs and 74% in cats, while the oral bioavailability in dogs and cats was 62% and 109%, respectively (Sarasola *et al.*, 2002). Moxidectin absorption is enhanced by co-administration of lipid in most species, but not in dogs (Lallemant *et al.*, 2007). Additionally within species, sex-related differences in absorption or bioavailability have been reported, including increased absorption of ivermectin and doramectin in female bovines (Toutain *et al.*, 1997).

MLs distribute widely throughout the body and tend to have long tissue residence times, resulting in prolonged antiparasitic activity. The degree of body fat can play a major role in the distribution of MLs, which in turn controls the terminal half-life of the individual animal; for instance, the elimination half-life of moxidectin in dogs with a high body fat content is longer than the half-life in leaner dogs (Lallemant *et al.*, 2007). ML levels in tissues such as the gastrointestinal mucosa, lungs and skin may exceed that of plasma for days to weeks following administration (Lanusse *et al.*, 2009). Doramectin achieves high concentrations in pulmonary tissue of cattle, making it highly effective against the lungworm *Dictyocaulus viviparus*. Following topical administration, selamectin is absorbed into the bloodstream and excreted via the bile into the intestinal tract, where it is reabsorbed and selectively distributed to the sebaceous glands in the skin (Gupta *et al.*, 2005; Gupta, 2007). This unique multiphasic distribution accounts for the ability of selamectin to control hematogenous, gastrointestinal and integumentary parasites following topical administration.

Although they have widespread distribution to most body tissues, MLs are largely excluded from the mammalian brain through the action of the BBB's p-glycoprotein transport system (P-GP) (Figure 51.7). The P-GP is a 170kDa trans-membrane protein coded for by the ABCB1 (formerly MDR1) gene and is located primarily in the intestine, liver, kidney and brain (Mosher and Court, 2010). The function of P-GP is to limit the systemic and brain uptake of certain xenobiotics and enhance their elimination from the body (Shen and Zhang, 2010). Alterations in P-GP function can result in increased systemic xenobiotic levels and enhanced penetration of xenobiotics into the brain. MLs comprise one family of drugs that serve as substrates for P-GP, therefore animals with defects within the P-GP system may have increased absorption and decreased elimination of MLs, as well as an influx of MLs into the CNS, where ML-sensitive GABA-gated chloride channels reside. Such animals will be hypersensitive to the neurologic effects of MLs at levels well below those tolerated by animals with intact P-GP and will show signs of toxicosis at relatively low levels. Alteration of P-GP function may occur via genetically mediated abnormalities or drug-drug interactions. The administration of drugs that alter P-GP function can also increase the sensitivity of an individual to the neurologic effects of MLs. P-GP inhibiting drugs such as ketoconazole can increase absorption and decrease elimination of MLs, increasing the likelihood of neurotoxicosis (Hugnet *et al.*, 2007).

In dogs, a 4 base-pair deletion resulting in a frame-shift mutation in the ABCB1 gene has been identified within several breeds (Mosher and Court, 2010). Affected dogs possess truncated, non-functional P-GP and are extremely sensitive to the effects of xenobiotics that serve as substrates for P-GP (Mealey, 2004). First identified in collies, the ABCB1 defect has also been identified in over 11 breeds of dogs, as well as mixes of these breeds (Geyer *et al.*, 2007; Mosher and Court, 2010). Table 51.1 lists the breeds in which ABCB1 defects have been identified. Interestingly, although increased brain penetration of xenobiotics has been demonstrated in dogs with the ABCB1 mutation, pharmacokinetic studies have shown no significant differences in oral bioavailability between dogs with mutant ABCB1 and wild-type dogs (Mealey *et al.*, 2010). Affected dogs develop clinical effects of ML toxicosis at levels that do not cause clinical signs in dogs with normal P-GP. Recently, a novel insertion mutation in the ABCB1 gene was found to be associated with ivermectin sensitivity in a border collie that lacked the previously described deletion mutation (Han *et al.*, 2010), suggesting that more than one genotypic abnormality may result in a similar phenotypic expression.

Although MLs undergo some metabolism, the majority are excreted unchanged in the feces via the bile. This excretion into the intestine results in reabsorption and enterohepatic recycling of up to 20% of the total dose of ML administered (Lanusse *et al.*, 2009). The fact that



**FIGURE 51.7** Cartoon of normal p-glycoprotein function within the blood–brain barrier (BBB). P-glycoprotein is a transmembrane pump (gray trapezoids) located on the apical (blood-facing) side of endothelial cells that form the BBB. These endothelial cells are joined through tight junctions (T.J.) that prevent diffusion of substances between the cells. (1) P-glycoprotein substrates (diamonds) diffuse into and out of the endothelial cells through the lipid bilayer of the cell membrane. (2) Substrates within the cell or cell membrane are taken up by the p-glycoprotein molecule, an active process requiring ATP for energy. (3) Once taken up by the p-glycoprotein, substrates are ejected back into the bloodstream. Defects in p-glycoprotein structure and/or function can result in increased concentrations of substrates within the endothelial cell, and egress of substrates out the basal side of the cell into the brain.

**TABLE 51.1** Dog breeds in which ABCB1 mutations have been identified

Australian shepherd
Border collie
Collie
English shepherd
German shepherd
Longhaired whippet
McNab
Old English sheepdog
Shetland sheepdog
Silken windhound
Wäller
White Swiss shepherd

Reported by Geyer *et al.* (2007); Mosher and Court (2010).

parenterally administered ML ultimately ends up being secreted into the bile and thereby enters the intestinal tract to undergo some degree of enterohepatic recycling largely explains the efficacy of injected MLs against intestinal parasites. Up to 95% of an ML dose is excreted unchanged in the feces and the half-lives for the various MLs are in the range of days to weeks rather than hours. Half-lives for the various MLs vary widely depending on formulation, dose administered and species, as well as the relative amount of body fat (Lo *et al.*, 1985; Fink and Porras, 1989; Lallemand *et al.*, 2007).

There have been many ML pharmacokinetic/toxicokinetic studies conducted in dogs, cats, sheep, goats, cattle, horse and pigs (Tway *et al.*, 1981; Lo *et al.*, 1985; Prichard *et al.*, 1985; Alvinerie *et al.*, 1987, 1993; Marriner *et al.*, 1987; Bogan and McKeller, 1988; Toutain *et al.*, 1988, 1997; Fink and Porras, 1989; Scott and McKeller, 1991; Godber *et al.*, 1995; Sarasola *et al.*, 2002; Gupta *et al.*, 2005). It is evident from many of these studies that ivermectin and its derivatives are excreted in the milk.

## MECHANISM OF ACTION

The MLs exert their toxic actions through their high affinity for ligand-gated chloride channels, particularly those mediated through the neurotransmitters gamma-aminobutyric acid (GABA) and glutamate, with higher concentrations required for GABA-mediated effects than for glutamate-mediated actions (Lanusse *et al.*, 2009). Glutamate-gated channels are unique to invertebrates (Trailovic and Nedeljovic, 2010). Binding of MLs to glutamate-gated chloride channels causes increased conductance of chloride through the cell membrane, resulting in hyperpolarization and flaccid paralysis of invertebrate musculature, particularly in nematodes and arthropods (Lanusse *et al.*, 2009). In particular, paralysis

of pharyngeal pump musculature decreases the ingestion of nutrients while paralysis of somatic musculatures impedes the ability of the parasite to remain at its site of predilection in the host, resulting in death of the parasites. Additionally, effects on the GABA and glutamate-mediated activity of the musculature of the female reproductive system results in decreases in ovi-position (Fellowes *et al.*, 2000).

Glutamate-mediated chloride channels sensitive to MLs are found uniquely in nematodes and arthropods. Cestodes and trematodes lack ML binding sites, and are therefore unaffected by MLs. In mammals, ML-sensitive GABA-mediated chloride channels are restricted to the CNS, from which the MLs are largely excluded through the action of a p-glycoprotein efflux pump (see MLs and p-glycoprotein defective animals in previous section). When the p-glycoprotein transporter is overwhelmed (e.g., in overdose situations), defective (e.g., ABCB1 defect) or compromised (e.g., pharmacologically inhibited) entry of MLs into the mammalian CNS may lead to signs of toxicosis.

## TOXICITY

### Toxicity data

At the doses used as parasiticides in veterinary medicine, MLs have a fairly low level of toxicity to the majority of animal species with at least a ten-fold margin of safety for ruminants, horses, swine and dogs with normal P-GP (Lanusse *et al.*, 2009). For example, the dosage of ivermectin used to prevent heartworms and intestinal nematodes in dogs is 6 µg/kg body weight, which is over 30 times less than the dosage of 200 µg/kg that is often used in dogs to manage ectoparasites such as *Demodex* mites. Dogs with ABCB1 defects would not be expected to show clinical signs until levels of 80–100 µg/kg of ivermectin were administered, while dogs with normal P-GP can generally tolerate doses of 200 µg/kg/day (Merola *et al.*, 2009). Toxic and therapeutic dosages for some macrolides are listed in Table 51.2.

TABLE 51.2 Toxicity of various macrocyclic lactone endectocides

Agent	Species	Therapeutic dose (route)	NOAEL	Toxic dose (LOEL or MTD unless otherwise stated)	Comments/References
Abamectin	Cattle	0.2 mg/kg, SC		2–8 mg/kg, SC	Not recommended for calves <4 mo; Lanusse <i>et al.</i> , 2009
	Dog (beagle)	N/A	0.25 mg/kg/d × 12 mo, PO		Stevens and Breckenridge, 2001
	Mouse			14–80 mg/kg (PO, LD <sub>50</sub> )	Exttoxnet, 1994 <sup>a</sup>
	Mouse (CF-1, pregnant)		0.05 mg/kg/day, PO	0.075 mg/kg/day, PO	Stevens and Breckenridge, 2001
	Mouse (CD-I)		4–8 mg/kg/day × 18 mo, PO		Stevens and Breckenridge, 2001
	Rabbit (pregnant)		1 mg/kg/day, PO	2 mg/kg/day, PO	Stevens and Breckenridge, 2001
	Rat	N/A		11 mg/kg (PO, LD <sub>50</sub> )	Exttoxnet, 1994 <sup>a</sup>
	Rat (SD)	N/A	1.5–2 mg/kg/d × 53 weeks, PO		Stevens and Breckenridge, 2001
	Rat (SD, pregnant)	N/A	1.6 mg/kg/day, PO	2.0 mg/kg/day PO	Stevens and Breckenridge, 2001
Doramectin	Camelids	0.2 mg/kg IM, SC			Fowler, 2010 <sup>b</sup>
	Cattle	0.2 mg/kg IM, SC	2.5 mg/kg, SC × 3 d		Lanusse <i>et al.</i> , 2009
		0.5 mg/kg, Topical			FDA, 1997 <sup>c</sup>
	Cattle (breeding)	0.2 mg/kg, SC	0.6 mg/kg, SC		Lanusse <i>et al.</i> , 2009
	Dogs (beagle)	N/A	2 mg/kg/day × 14 d		INCHEM, 2010 <sup>d</sup>
	Dogs (collie)	N/A	0.25 mg/kg, PO	0.5 mg/kg, PO	INCHEM, 2010 <sup>d</sup>
	Goats	0.2 mg/kg IM, SC			Pfizer, 2006 <sup>e</sup>
	Pigs	0.3 mg/kg IM	3 mg/kg, IM	7.5 mg/kg, IM	Lanusse <i>et al.</i> , 2009; EMEA, 1997 <sup>f</sup>
	Rats	0.2 mg/kg q 24 h × 4 d, PO			Oege <i>et al.</i> , 2000 <sup>g</sup>
	Sheep	0.2 mg/kg IM, SC	0.6 mg/kg, SC		Lanusse <i>et al.</i> , 2009; EMEA, 1997 <sup>f</sup>

(Continued)



TABLE 51.2 (Continued)

Agent	Species	Therapeutic dose (route)	NOAEL	Toxic dose (LOEL or MTD unless otherwise stated)	Comments/References
Eprinomectin	Camelids	0.5 mg/kg, topical			Plant <i>et al.</i> , 2007 <sup>h</sup>
	Cattle	0.5 mg/kg, topical	2.5 mg/kg, topical		Lanusse <i>et al.</i> , 2009
	Dogs	N/A	1 mg/kg/d × 14 d, PO	1.6 mg/kg/d × 14 d, PO	EMA, 1996 <sup>i</sup>
	Horses	0.5 mg/kg, topical			Ural <i>et al.</i> , 2008 <sup>j</sup>
	Goats	1 mg/kg, topical			Scheuerle <i>et al.</i> , 2009 <sup>k</sup>
	Mice	N/A		75 mg/kg, PO (LD <sub>50</sub> ), 35 mg/kg, IP (LD <sub>50</sub> )	EMA, 1996 <sup>i</sup>
	Rats	N/A	5 mg/kg/d × 14 d, PO	55 mg/kg, PO (LD <sub>50</sub> ), 35 mg/kg, IP (LD <sub>50</sub> )	EMA, 1996 <sup>i</sup>
	Sheep	0.5 mg/kg, topical			Kircali Sevimli <i>et al.</i> , 2007 <sup>l</sup>
Ivermectin	Camelids	0.2–0.6 mg/kg, SC			Fowler, 2010 <sup>b</sup>
	Cats	0.024–0.4 mg/kg, PO	0.75 mg/kg, PO	1.0 mg/kg, PO	FDA, 1996 <sup>m</sup> ; Lanusse <i>et al.</i> , 2009
	Cattle	0.5 mg/kg, topical, 0.2 mg/kg, SC	5 mg/kg, topical	0.6 mg/kg, SC	Lanusse <i>et al.</i> , 2009; Gupta, 2007
	Dogs	0.006–0.6 mg/kg, PO		80 mcg/kg (ABCB1 defective) 0.2–1 mg/kg (ABCB1 normal)	Plumb, 2009 <sup>n</sup> ; Merola <i>et al.</i> , 2009
	Goats	0.4 mg/kg, PO			Lanusse <i>et al.</i> , 2009
	Horses	0.2 mg/kg, PO		1.2–2 mg/kg, PO	Lanusse <i>et al.</i> , 2009; Gupta, 2007
	Pigs	0.1 mg/kg/d × 7 d, PO			Lanusse <i>et al.</i> , 2009
	Sheep	0.2 mg/kg, PO			Lanusse <i>et al.</i> , 2009
Milbemycin	Cats	2 mg/kg, PO			Lanusse <i>et al.</i> , 2009
	Dogs	0.5 mg/kg, PO	0.6 mg/kg (ABCB1 defective)	5 mg/kg (ABCB1 defective)	Lanusse <i>et al.</i> , 2009; Barbet <i>et al.</i> , 2009 <sup>n</sup> ; Tranquilli <i>et al.</i> , 1991 <sup>o</sup>
					Lanusse <i>et al.</i> , 2009
Moxidectin	Cattle	0.2–1 mg/kg SC, Topical	2 mg/kg		Lanusse <i>et al.</i> , 2009
	Dogs	0.003–0.17 mg/kg, PO			Lanusse <i>et al.</i> , 2009
	Horses	0.4 mg/kg, PO		1 mg/kg	Lanusse <i>et al.</i> , 2009; Khan <i>et al.</i> , 2002 <sup>p</sup>
	Pigs	0.3 mg/kg, SC			Lanusse <i>et al.</i> , 2009
	Sheep	0.2–1 mg/kg PO, SC			Lanusse <i>et al.</i> , 2009
Selamectin	Cats	6 mg/kg, topical	60 mg/kg, topical		Lanusse <i>et al.</i> , 2009
	Dogs	6 mg/kg, topical	60 mg/kg, topical		Lanusse <i>et al.</i> , 2009

<sup>a</sup>ExToxNet (1994) Abamectin. Pesticide Information Profile. Cornell University. Accessed via web 2/2011. <http://pmep.cce.cornell.edu/profiles/extoxnet/24d-captan/abamectin-ext.html>

<sup>b</sup>Fowler ME (2010) Parasites. In *Medicine and Surgery of Camelids*, Fowler ME (ed.). Wiley Blackwell, p. 259.

<sup>c</sup>FDA (1997) Dectomax® Approval Letter, FOIA.

<sup>d</sup>INCHEM (2010) International Programme on Chemical Safety. Accessed via web 2/2011. <http://www.inchem.org/documents/jecfa/jecmono/v36je02.htm>

<sup>e</sup>Pfizer (2006) Dectomax® Label Information. Pfizer Animal Health, New York.

<sup>f</sup>EMA (1997) Doramectin (pigs and sheep). European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit. EMA/MRL/114/96-FINAL. Accessed via web 3/2011: [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Maximum\\_Residue\\_Limits\\_-\\_Report/2009/11/WC500013916.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500013916.pdf)

<sup>g</sup>Oege H, Ayaz E, Dalgic S (2000) The effect of doramectin, moxidectin and netobimbin against natural infections of *Syphacia muris* in rats. *Vet Parasitol* **88**: 299–303.

<sup>h</sup>Plant JD, Kutzler MA, Cebra CK (2007) Efficacy of topical eprinomectin in the treatment of *Chorioptes* sp. infestation in alpacas and llamas. *Vet Dermatol* **18**: 59–62.

<sup>i</sup>EMA (1996) Eprinomectin. European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit. EMA/MRL/114/96-FINAL. Accessed via web 3/2011: [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Maximum\\_Residue\\_Limits\\_-\\_Report/2009/11/WC500014177.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014177.pdf)

<sup>j</sup>Ural K, Ulutas B, Kar S (2008) Eprinomectin treatment of psoroptic mange in hunter/jumper and dressage horses: a prospective, randomized, double-blinded, placebo-controlled clinical trial. *Vet Parasitol* **156**: 353–357.

<sup>k</sup>Scheuerle MC, Mahling M, Pfister K (2009) Anthelmintic resistance of *Haemonchus contortus* in small ruminants in Switzerland and Southern Germany. *Wien Klin Wochenschr* **121**: 46–49.

<sup>l</sup>Kircali Sevimli F, Kozan E, Dolan N (2011) Efficacy of eprinomectin pour-on treatment in sheep naturally infected with *Dictyocaulus filaria* and *Cystocaulus ocreatus*. *J Helminthol* **7**: 1–4.

<sup>m</sup>FDA (1996) Heartgard® for Cats Original Approval. Accessed via web: <http://www.fda.gov/AnimalVeterinary/Products/ApprovedAnimalDrugProducts/FOIADrugSummaries/ucm116793.htm>

<sup>n</sup>Barbet JL, Snook T, Gay JM, Mealey KL (2009) ABCB1-1 Delta (MDR1-1Delta) genotype is associated with adverse reactions in dogs treated with milbemycin oxime for generalized demodicosis. *Vet Dermatol* **20**: 111–114.

<sup>o</sup>Tranquilli WJ, Paul AJ, Todd KS (1991) Assessment of toxicosis induced by high-dose administration of milbemycin oxime in collies. *Am J Vet Res* **52**: 1170–1172.

<sup>p</sup>Khan SA, Kuster DA, Hansen SR (2002) A review of moxidectin overdose cases in equines from 1998–2000. *Vet Hum Toxicol* **44**: 232–235.

## Clinical effects

The clinical signs of ivermectin toxicosis in collies have been reported in several studies (Pullium *et al.*, 1985; Paul *et al.*, 1987; Tranquilli *et al.*, 1987). Ivermectin is a neurotoxicant, as it exerts its toxic effects on the CNS of these sensitive dogs. Further support for CNS involvement was the finding that the ivermectin concentrations in the brain were higher in dogs with P-GP defects displaying signs of ivermectin toxicosis than in dogs with normal P-GP (Pullium *et al.*, 1985). These findings not only suggest involvement of the CNS, but also a difference in the BBB permeability to ivermectin in sensitive versus non-sensitive dogs. However, no differences in the binding characteristics of ivermectin in plasma (albumin and the high density lipoprotein) from ivermectin-sensitive and non-sensitive collies were found (Rohrer and Evans, 1990). The difference between sensitive and non-sensitive dogs lies within the anatomy or physiology of BBB itself or in the presence of high affinity binding sites within the brain rather than difference in the bioavailability of the drug (Kitagawa *et al.*, 1988; Sasaki *et al.*, 1988; Tranquilli *et al.*, 1989). A collie after ingesting ivermectin at about 200 µg/kg (in an equine anthelmintic paste) exhibited the signs of dehydration, bradycardia, respiratory depression, cyanosis, dilated pupils and a diminished gag reflex (Heit *et al.*, 1989). Toxic signs may also include vomiting, ataxia, tremors, hypersalivation, coma and death. Toxic reactions in cats receiving ivermectin (500 µg/kg, SC) include signs such as vocalization, ataxia, tremors, sternal recumbency, coma and death. Young kittens are at increased risk for toxicosis due to increased permeability of their immature BBB. Kittens with ivermectin toxicosis developed signs of mild diarrhea, posterior ataxia and mild miosis (Song, 1991). In contrast, in mammals in which ivermectin does not readily cross the BBB, the drug has a wide margin of safety and at least ten times the therapeutic dose is required to produce toxic effects in most normal mammals (Campbell *et al.*, 1983; Bennett, 1986).

Ivermectin given IV or SC to 8-month-old Jersey bull calves at 600 µg/kg caused depression, ataxia, diarrhea, dyspnea, tachycardia and miosis. Signs were less severe after SC than they were after IV injection. Some of these signs were associated with GABA-mediated cholinergic effects. Calves receiving 8.0 mg/kg developed ataxia and recumbency within 24 h after ivermectin exposure. Calves developed depression, increased respiratory rates, muscular fasciculations, mydriasis and extensor rigidity of the limbs. In cattle, the maximum tolerated dose of B<sub>1a</sub> is about 1.0 mg/kg. With a higher dose of ivermectin in sheep, toxicosis is characterized by depression and incoordination.

Horses exposed to higher doses of ivermectin (six to ten times greater than the therapeutic dose) showed

ataxia, depression and vision impairment within 24 h. With a dose of 60 times the recommended dosage (12 mg/kg), horses showed depression, mydriasis, ataxia, depressed respiratory rate and drooping lower lip (Leaning, 1983).

In an experimental study, Wise *et al.* (1997) demonstrated that at a high dose, emamectin benzoate (derivative of avermectin B<sub>1</sub>) exposure during gestation and lactation in rats produced evidence of neurotoxicity in the F<sub>1</sub> offspring. A no observed adverse effect level for developmental neurotoxicity of emamectin was determined to be 0.6 mg/kg/day.

In acute selamectin poisoning cases, signs of toxicosis may include hair loss at the site of application, vomiting, diarrhea with or without blood, anorexia, lethargy, salivation, tachypnea, pruritis, urticaria, erythema, ataxia and fever. In rare instances, seizures followed by death occur with overt acute overdose. In chronic cases, in dogs and cats, seven monthly treatments of 60 mg/kg (ten times the recommended dose) produced no adverse reactions when given to 6-week-old kittens or puppies. An exposure of 18 mg/kg (three times recommended dose) produced no effect on reproduction in females or males. Three monthly doses of 30 mg/kg produced no adverse effects in ivermectin-sensitive collies. There have also been rare reports of muscle spasms, seizures, ataxia and other neurological signs. In a recent study, dogs treated with a single topical application of Revolution™ (6 mg selamectin/kg body weight) showed no signs of poisoning (Gupta *et al.*, 2005).

## Diagnosis

Diagnosis of ivermectin, selamectin and other MLs can be based on history of exposure to a product, clinical signs and residue analysis in the body tissue or fluids. These compounds are analyzed using high performance liquid chromatograph coupled with a UV, fluorescence or photo diode array detector (Reising *et al.*, 1988; Maynard and Maynard, 1989; Rabel *et al.*, 1993; Payne *et al.*, 1995; Anastaseo *et al.*, 2002; Gupta *et al.*, 2005). GI content, liver, fat and feces are usually the specimens analyzed for MLs residue.

## TREATMENT

Treatment for intoxication with MLs should include limiting systemic absorption of the xenobiotic, monitoring for possible clinical effects and managing any signs that develop. Symptomatic patients should be stabilized prior to performing decontamination procedures. For

oral exposures, emesis, lavage and/or administration of activated charcoal should be considered. If the exposure was by a recent subcutaneous injection and life-threatening toxicosis is possible, surgical excision of the injection site can be considered if the bleb can still be palpated (Beasley *et al.*, 1999).

For patients that can safely vomit, emesis may be effective if performed within 2 hours of ingestion (Mealey, 2006). If the patient is unable to safely vomit (for example, is a species that cannot vomit or is symptomatic), gastric lavage can be considered. Activated charcoal should be considered in addition to or instead of emesis or lavage. It is important to mention that with xenobiotics, like the MLs, which are excreted in the bile, activated charcoal can be of benefit regardless of the route of the exposure. Thus if a patient received an overdose of injectable ML subcutaneously, activated charcoal will still be a very valuable decontamination option. The ML molecules will be carried to the gastrointestinal tract by the bile.

MLs undergo enterohepatic recycling therefore multiple doses of activated charcoal will likely be beneficial. In addition, the MLs are substrates for the p-glycoprotein transport system (P-GP) that transports some drugs across cell membranes. In the intestine, the MLs enter the enterocyte by absorption from the gastrointestinal tract. However, once in the cell, the P-GP acts to move the ML across the membrane and back into the gastrointestinal lumen. This cycling allows the ML molecules to have multiple opportunities to bind with the repeated doses of activated charcoal (Mealey, 2006). When repeated doses are indicated, half the original dose should be given at 4 to 8 hour intervals, often for 2–3 days (Peterson, 2006).

The patient should be monitored for the development of CNS effects including ataxia, lethargy, recumbency, tremors and seizing. Also monitor for bradycardia, gastrointestinal upset and respiratory depression. No specific chemistry panel changes are expected. If activated charcoal is given, serial serum sodium tests should be run to check for elevations as hyponatremia has been associated with repeated doses of activated charcoal.

There is no specific antidote for ML toxicosis so treatment is symptomatic and supportive. Patients who are recumbent or comatose will require good nursing care including thermoregulation, soft bedding and frequent turning to prevent decubital ulcers and urine scalding since the patient may be immobile for multiple days (Mealey, 2006). Patients that are experiencing tremors or seizures can be treated with methocarbamol. Minimize sensory stimuli since these patients can be hyperesthetic. Nutritional support through tube feeding may also be necessary (Mealey, 2006). Intravenous fluids should be given as needed for cardiovascular support and atropine can be used for bradycardia. A respirator may be

necessary if significant respiratory depression develops. Picrotoxin and physostigmine have both been used to treat ivermectin toxicosis but have been associated with significant adverse effects including seizures (Crandell and Weinberg, 2009).

A promising new therapy, intravenous infusion of a lipid emulsion (ILE), has been used to treat moxidectin toxicosis in a puppy (Crandell and Weinberg, 2009). Lipid emulsions are made from purified soybean oil in water, are commonly used in medicine to provide intravenous (parenteral) nutrition and are the delivery mechanism for certain hydrophobic drugs like propofol.

The mechanism by which ILE is effective at treating toxicoses is not yet fully understood. However, the fact that ILE seems to be most effective in treating overdose of lipid soluble medications suggests that the infusion expands the amount of plasma lipid which acts as a sink in which the offending xenobiotic can gather thus reducing free drug concentrations. In theory the xenobiotic is trapped in the plasma lipid so it is not available to act on other tissues (Crandell and Weinberg, 2009; O'Brien *et al.*, 2010).

The ASPCA Animal Poison Control Center uses the following dosing protocol for ILE. Using a 20% product, give an initial bolus of 1.5 ml/kg slowly then start a continuous rate infusion (CRI) of 0.25 ml/kg/min for 30–60 minutes. Four hours after the CRI is finished, check the serum for hyperlipemia and to see if the serum is orange or yellow. If the serum looks normal, repeat the initial bolus and CRI again. If hyperlipemia or a color change is present, check the serum for resolution every 2 hours. Repeat the initial bolus and CRI once the hyperlipemia or color change resolves. If a third dose is needed, follow the above directions beginning 4 hours after the second CRI finishes. Do not give more than three doses if there has been no significant response.

Possible side effects of administering ILE include induction of pancreatitis, creation of a fat embolism, immunosuppression, phlebitis, thrombosis, hypertriglyceridemia and hepatic lipidosis (Crandell and Weinberg, 2009; O'Brien *et al.*, 2010).

## CONCLUSIONS

Macrocyclic lactones (MLs) are commonly used as insecticides, nematicides and acaricides in animals. Among all MLs, acute toxicity is more often encountered with ivermectin. Poisoning occurs in dogs (especially collies) and cats due to inadvertent or misuse of the product meant for another species. In general, young animals are affected with a greater frequency than adults. Clinical signs are those of CNS toxicity. Treatment relies upon symptomatic and supportive therapies.

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# Rotenone

Ramesh C. Gupta

## INTRODUCTION

Rotenone is one of the oldest naturally occurring compounds present in a number of plants. Rotenone is the trivial name of the main chemical component of certain plants of the “Derris,” “*Lonchocarpus*,” “*Tephrosia*” and “*Mundulea*” species. It has a molecular formula of  $C_{23}H_{22}O_6$  and a molecular weight 394.42. Its chemical structure is shown in Figure 52.1. Rotenone is present in the form of colorless crystals, but is readily oxidized by light, and becomes yellow, orange and then deep red. Rotenone and its formulatory products have other names, such as Barbasco, Chem-Fish, Cubé, Cuberol, Derris, FishTox, Haiari, Nicouline, PrenFish, Prentox, Rotacide, SprenFish, Tubatoxin and Tox-R. It is also marketed as Control Garden Dust, Chem-Mite, Cibe Extract, Curex Flea Dust, Derrin and Green Cross Warble Powder. Rotenone is used worldwide because it has broad spectrum insecticidal, acaricidal and piscicidal properties.

Rotenone is also formulated along with other pesticides such as carbaryl, pyrethrins, piperonyl butoxide, lindane and others in products to control insects, mites, ticks, lice, spiders and undesirable fish. In general, rotenone is an excellent organic pesticide used in home gardens for insect control, for lice and ticks on pets, and fish eradications as part of water body management. In veterinary medicine, rotenone is used in powder form to control parasitic mites on chickens and other fowl, and for lice and ticks on dogs, cats and horses. According to a survey conducted by the EPA in 1990, rotenone was found to be one of the pesticides most commonly used in and around the home. During the past decade, rotenone has received enormous attention because of its link

to Parkinson’s disease (Tanner *et al.*, 2011). Rotenone has been found toxic to humans, animals and fish. This chapter describes the toxicity of rotenone in animals.

## BACKGROUND

Emmanuel Geoffroy isolated nicouline (later termed rotenone) for the first time from a specimen of *Robinia nicou*, now called *Lonchocarpus nicou*. Rotenone and rotenoids have been used as crop insecticides since 1848, when they were applied to plants to control leaf eating caterpillars (Ware, 1994). These compounds have been used for centuries (since 1649) in South America to paralyze fish, causing them to surface. Rotenone and rotenoids are extracted mainly from the roots, but also from the seeds and leaves, of certain plants grown in Malaya, East Indies and Central and South America. Presently, Peru is the major source of the root of the plant, which may be ground as a dust or extracted to provide concentrates. Other rotenone-related compounds such as deguelin, tephrosin and toxicarol have been isolated from various parts of rotenone-containing plants, such as legume shrubs. Rotenone has been a registered pesticide in the U.S. under the Federal Insecticide Fungicide Rodenticide Act (FIFRA) since 1947. It is a selective and non-specific insecticide with some acaricidal properties. Its formulations include crystalline preparations (about 95%), emulsifiable solutions (about 50%) and dust (0.75%). Rotenone dust is used for insects, lice and ticks on animals; and beetles and aphids on vegetables, fruits, berries and flowers. Rotenone emulsions are used for eliminating unwanted fish in the management of bodies of water.

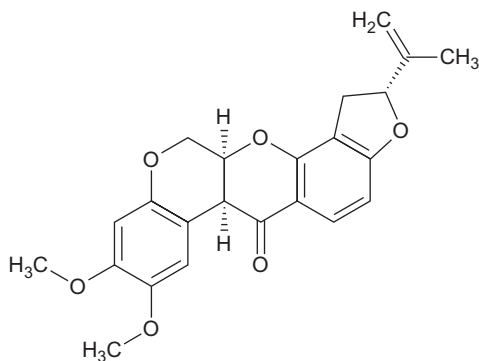


FIGURE 52.1 Chemical structure of rotenone.

Rotenone is a very safe compound when properly used, but in higher doses it is toxic to humans, animals and fish. Rotenone has been involved in suicide attempts, in which acute congestive heart failure was the characteristic feature at autopsy. Currently, rotenone is extensively used by researchers as an experimental drug to produce mitochondrial dysfunction and reproduce Parkinson's disease in animal models (Moon *et al.*, 2005; Borland *et al.*, 2008; Watabe and Nakaki, 2008; Drolet *et al.*, 2009). Chronic exposure to rotenone has also been associated with mitochondrial dysfunction, oxidative stress and pathophysiology of Parkinson's disease in humans (Tanner *et al.*, 2011).

## PHARMACOKINETICS/ TOXICOKINETICS

Absorption of rotenone from the gastrointestinal (GI) tract is low and incomplete. In animals, rotenone has been found to be hundreds of times more toxic via the IV route than by the oral route. Fats and oils increase the absorption of rotenone from the GI tract. Rotenone is metabolized in the liver by NADP-linked hepatic microsomal enzymes. Several metabolites have been identified as rotenoids, such as rotenolone I and II, hydroxyl and dihydroxyrotenones, etc. (Hayes, 1982; Gosselin *et al.*, 1984). It has been reported from studies conducted on rats and mice that approximately 20% of a dose is excreted in the urine within 24h of oral administration (Hayes, 1982). Unabsorbed rotenone from the GI tract is excreted in the feces.

## MECHANISM OF ACTION

In insects, rotenone is both a contact and a systemic insecticide. It is used as a broad-spectrum insecticide

that works by inhibiting the transfer of electrons from Fe-S centers in complex I to ubiquinone in the electron transport chain. This prevents NADH from being converted into usable cellular energy, i.e., ATP.

In mammals and fish, rotenone inhibits the oxidation of NADH to NAD, thereby blocking the oxidation of NAD and the substrates such as glutamate,  $\alpha$ -ketoglutarate and pyruvate. In *in vitro* studies, Hayes (1982) demonstrated that the aerobic oxidation of pyruvic acid was completely inhibited by rotenone in isolated rat liver mitochondria. Rotenone causes inhibition of mitochondrial respiratory chain complex I, and cell death by apoptosis due to excess generation of free radicals (Li *et al.*, 2003; Nianyu *et al.*, 2003; Watabe and Nakaki, 2008; MacKenzie *et al.*, 2008). In addition, rotenone causes a definite anesthetic effect when it comes in contact with nerve axons (Hayes, 1982). Following chronic exposure to rotenone, fatty acid synthesis is altered in the mitochondria, resulting in fatty changes in the liver and kidney (Hayes, 1982; Windholz, 1983; Gosselin *et al.*, 1984).

## TOXICITY

Rotenone is a naturally occurring chemical with insecticidal, acaricidal and fish-killing properties. Rotenone is toxic to humans as well as animals. WHO classifies rotenone as a moderately hazardous class II pesticide.

In mammals, the acute toxicity of rotenone is moderate and widely varies between and within species (Ellenhorn and Barceloux, 1988). The oral LD<sub>50</sub> value of rotenone in rats is approximately 60–135 mg/kg body weight, while in mice the value is approximately 350 mg/kg. The LD<sub>50</sub> IP in mice is 2.8 mg/kg. Rotenone is more toxic to female rats than males. Rotenone is less toxic in mice and hamsters than in rats. Pigs seem to be especially sensitive. In rabbits, the LD<sub>50</sub> values for rotenone following IV, oral and dermal routes are 0.35–0.65, 1.5 and 100–200 mg/kg, respectively.

In general, depression and convulsions are the common clinical signs with acute toxicity of rotenone. Following oral ingestion, clinical signs of rotenone toxicosis may include pharyngitis, nausea, vomiting, gastric pain, clonic convulsions, muscle tremors, lethargy, incontinence and respiratory stimulation followed by depression (Hayes, 1982; Gosselin *et al.*, 1984). In animals, severe signs of hypoglycemia and liver failure are evident. Rotenone causes alterations in arterial blood gases and acid-base balance. It induces hypoxemia and hypercapnia due to respiratory depression and seizures. In addition, rotenone can impair myocardial contractile force.

Inhalation of rotenone dust is known to cause severe pulmonary irritation and asphyxia. In rats and dogs, experimental inhalation of rotenone dust produced onset of signs earlier than following oral ingestion (Hayes, 1982). Toxicity is greater if the particles are of smaller size, because these particles can enter the deep regions of the lungs.

Studies suggest that following parenteral administration, rotenone can induce vomiting, incoordination, muscle tremors, clonic convulsions and respiratory failure. Terminal symptoms of rotenone poisoning are convulsions and cardio-respiratory failure (Gosselin *et al.*, 1984; Ellenhorn and Barceloux, 1988). Most often, cardiovascular effects include tachycardia, hypotension and impaired myocardial contractility.

Rotenone is a neurotoxicant. In the rat model, IV infusion of rotenone (2–3 mg/kg/day) is known to produce Parkinson's-like pathology. Both *in vivo* and *in vitro* studies have demonstrated that chronic exposure to rotenone results in degeneration of neuronal cells, especially dopaminergic neurons, and is thereby responsible for the development of the signs of Parkinson's disease (Li *et al.*, 2003; Moon *et al.*, 2005; Watabe and Nakaki, 2008; MacKenzie *et al.*, 2008). The observed neurodegenerative effects are due to inhibition of mitochondrial complex I activity and enhanced production of reactive oxygen species and nitric oxide, leading to excess formation of the toxic factor, peroxynitrite. Chronic rotenone exposure also reproduced Parkinson's disease-like gastrointestinal neuropathology in rats and mice (Drolet *et al.*, 2009; Pan-Montojo *et al.*, 2010).

In a chronic study, dogs receiving rotenone (10 mg/kg/day) for 6 months showed weight loss and hematological changes. Chronic exposure to rotenone may produce fatty changes in the liver and kidney (Windholz, 1983). A no observed adverse effect level (NOAEL) of 0.4 mg/kg/day has been determined in rats and dogs.

Rotenone has no potential for endocrine disruption, but it does have the potential for reproductive toxicity and teratogenicity. Pregnant rats fed 5 mg/kg/day produced a significant number of young with skeletal deformities. In guinea pigs, fetotoxicity has been observed at doses of 4.5 and 9.0 mg/kg/day. Studies suggest that rotenone has no potential to induce mutagenicity (Waters *et al.*, 1982; Moriya *et al.*, 1983). Rotenone may produce tumors in vitamin-deficient animals (Gosalvez, 1983). Its potential to cause carcinogenicity remains controversial.

Rotenone has selective toxicity, as it is highly toxic to fish because of its rapid absorption from the GI tract in comparison to mammalian species in which it is poorly absorbed. The selective toxicity of rotenone in insects and fish versus mammals can also be explained based on the metabolism of this compound. Rotenone converts to highly toxic metabolites in large quantities in insects and fish, while it converts to nontoxic metabolites in mammals.

## Diagnosis

Cases of rotenone poisoning in animals are rare. Diagnosis can be based on circumstantial evidence and detection of rotenone residue in blood, urine, feces and vomitus. It is expected that rotenone residue is more likely to be present in the liver. Rotenone residue can be determined using HPLC coupled with a fluorescence detector or LC-MS-MS (Caboni *et al.*, 2008).

## TREATMENT

There is no specific antidote available for rotenone or rotenoids. Treatment relies upon symptomatic and supportive measures (including oxygen and artificial respiration). Wash contaminated skin to decontaminate rotenone residue. Avoid emesis if convulsions are present or the ingested product has petroleum distillate. Perform gastric lavage, followed by the administration of a slurry of activated charcoal in water. Control seizures and agitation with diazepam and correct hypoglycemia with glucose (5%, IV).

## CONCLUSIONS

Rotenone is widely used as insecticide, acaricide and piscicide, throughout the world. It has selective toxicity, i.e., highly toxic to insects and fish in comparison to mammalian species. Cases of rotenone poisoning in animals are rare. Rotenone causes mitochondrial dysfunction and oxidative stress and cell death occurs by apoptosis. Rotenone is a neurotoxicant and death ensues due to cardiac-respiratory failure. Treatment relies upon symptomatic and supportive therapy.

## ACKNOWLEDGMENTS

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# Metaldehyde

Ramesh C. Gupta

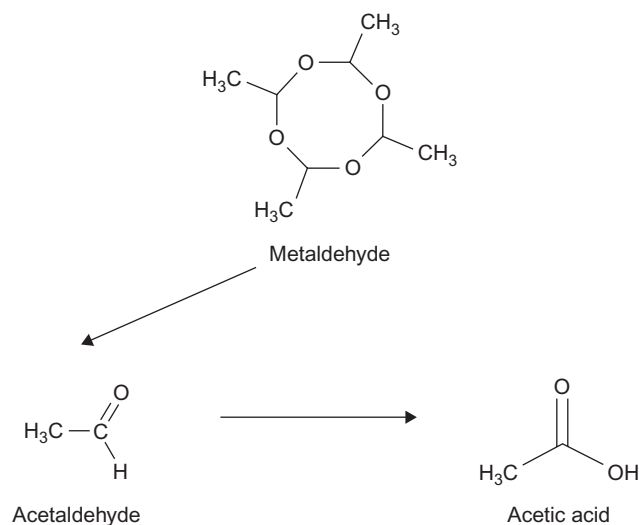
## INTRODUCTION

Metaldehyde (2,4,6,8-tetramethyl-1,3,5,7-tetraoxacyclooctane) is a cyclic polymer of acetaldehyde (Figure 53.1), which has a chemical formula of  $C_8H_{16}O_4$  and a molecular weight of 176. It is found in the form of a white or colorless crystalline solid or powder. Metaldehyde is used primarily as a molluscicide to control slugs and snails in a very wide range of gardens and croplands worldwide. Some common slug and snail baits containing metaldehyde include "Cory's Slug and Snail Death," "Deadline" and "Slug-Tox." These products are sold as granules, sprays, dusts, pelleted grain or bait. Metaldehyde is also used in many pesticide products along with other compounds, such as carbamates (carbaryl, cloethocarb or methiocarb), organophosphates and arsenates. Secondary use of this pesticide is for the control of fish, leeches and frogs. Metaldehyde, in combination with a carbamate pesticide, is also applied as a seed dressing in farm crops, such as wheat. In addition, metaldehyde is a main ingredient of solid fuel used for making camp fires (fire starter; camp fuel for portable stove) or in small heaters, and is also marketed as a color flame tablet for party goods (ENGELFIRE). Oral ingestion of metaldehyde in the forms of molluscicides and fuel tablets is the most common source of exposure. Poisoning incidents have been documented in pets (Studdert, 1985; Booze and Oehme, 1985, 1986; Dolder, 2003; Yas-Natan *et al.*, 2007; Campbell, 2008), domestic and wild animals (Harris, 1975; Stubbings *et al.*, 1976; Sutherland, 1983; Fletcher *et al.*, 1999; Valentine *et al.*, 2007; Daniel *et al.*, 2009) and birds (Andreasen, 1993). Poisoning incidents with metaldehyde are most frequently encountered in dogs and cats. Metaldehyde has also been involved in accidents and suicide attempts

(Longstreth and Pierson, 1982; Shih *et al.*, 2004; Bleakley *et al.*, 2008). This chapter describes the toxicity of metaldehyde and its metabolite acetaldehyde in mammalian and avian species.

## BACKGROUND

Metaldehyde has been used as a molluscicide since 1936. Currently, it is the most popular molluscicide used around the world in a variety of vegetables and agricultural crops, greenhouses, gardens and lawns and turf. About 48,000 pounds of metaldehyde are used in the United States each year. It is applied in liquid form, granules, sprays, dusts or pelleted/grain bait to kill slugs, snails and other garden pests. Usually, the commercial baits contain 4% or less metaldehyde as active ingredient. Some of the granule baits can have up to 5–10% metaldehyde. In Europe, the bait can have up to 50% metaldehyde. These products are formulated with a blue or green color. Baits formulated in pellets resemble dog food and are flavored with bran or molasses to attract snails. Unfortunately, the flavor attracts dogs and other pets as well. Acute poisoning is common in pets, birds and domestic and wild animals. A very small amount of metaldehyde is required to cause poisoning or death. The major target organs for metaldehyde toxicity include CNS, liver, kidney and lung. Animals that ingest metaldehyde may exhibit a variety of toxicological signs including vomiting, tachycardia, ataxia, tremors, seizures, coma and death (Dolder, 2003). Upon dermal contact, metaldehyde may cause irritation. Currently, there is an increase in the number



**FIGURE 53.1** Chemical structures of metaldehyde and its two major metabolites (acetaldehyde and acetic acid).

of metaldehyde poisoning cases in dogs attributed to accidental/inadvertent or malicious use of this chemical in baits (Campbell, 2008). This is in spite of the fact that slug baits containing metaldehyde have denatonium benzoate, commonly known as Bitrex (bitter-tasting substance) to help reduce the number of poisonings.

## TOXICOKINETICS

Metaldehyde and its metabolites are readily absorbed from the GI tract. Metaldehyde can also be absorbed from the lungs and skin. Following oral ingestion, maximal concentrations in the circulation and tissues can be attained within 1–3 h. Metaldehyde secretes back into the GI tract because it gets trapped in enterohepatic circulation (Sax, 1984; Dreisbach, 1987; Knowles, 1991). Residues of metaldehyde have been detected in the brain, blood and liver of mice (NLM, 1995; Puschner, 2006).

Metaldehyde undergoes hydrolysis in the acidic pH of stomach, forming acetaldehyde as a major degradation product. Acetaldehyde is then oxidized to acetic acid (Figure 53.1). The half-life of metaldehyde at 24°C is 0.75 and 4 h, in 0.1 and 0.01 mol/L aqueous hydrochloric acid, respectively (Sparks *et al.*, 1996). Following absorption, a significant amount of metaldehyde is metabolized by cytochrome P450. Metaldehyde, but not acetaldehyde, was found in the plasma and urine of dogs given a single oral dose of 600 mg/kg body weight (Booze and Oehme, 1986). Similar findings were reported in a human case (Moody and Inglis, 1992). Metaldehyde can be excreted in urine and feces. Tardieu *et al.* (1996) reported that

only 8% of an oral dose of metaldehyde was excreted as unmetabolized in the urine and feces. Urinary excretion was found to be less than 1% of the oral dose (600 mg/kg, PO) administered to dogs (Booze and Oehme, 1986). Its elimination half-life is reported to be 27 h (Olson, 1999), so its residue does not build up in the body.

## MECHANISM OF ACTION

The pesticidal action of metaldehyde in mollusks and snails is due to direct contact, making them torpid and increasing the secretion of mucus leading to fatal dehydration, convulsions and paralysis (RSC, 1987).

In mammalian and avian species, the toxicity of metaldehyde is characterized by CNS signs, metabolic acidosis and respiratory alkalosis. The exact mechanisms involved are yet to be elucidated. The proposed mechanism of action is that acetaldehyde, which is formed from metaldehyde at a low pH in the stomach, is responsible for the toxic effects observed with metaldehyde exposure (Knowles, 1991). Other toxic products are probably also formed. Metaldehyde and its metabolite acetaldehyde can cross the blood–brain barrier, as evidenced by their neurotoxic effects in animals (NLM, 1995). Acetaldehyde acts as a releasing factor for 5-hydroxytryptamine (5-HT) and norepinephrine. CNS signs of metaldehyde toxicity may be due to decreased brain concentrations of  $\gamma$ -aminobutyric acid (GABA), norepinephrine and 5-HT, as well as increased monoamine oxidase (MAO) activity (Homeida and Cook, 1982a,b; Dolder, 2003). Acetaldehyde competitively inhibits biogenic amine oxidation, which in turn decreases 5-hydroxyindoleacetic acid, a metabolite of 5-HT. Acetaldehyde also increases MAO activity and decreases central 5-HT levels (Booze and Oehme, 1985). The old theory that the toxicity of metaldehyde is attributable to its metabolite acetaldehyde is questionable, because acetaldehyde was not present in the plasma and urine of dogs or the serum of rats that were given metaldehyde (Shintani *et al.*, 1999; Puschner, 2006). Metaldehyde also affects electrolyte and acid–base balances, which can cause metabolic acidosis and respiratory alkalosis that is often associated with central nervous system depression and hyperpnea (Puschner, 2006).

## TOXICITY

Metaldehyde is toxic to all mammals and birds, but not to aquatic life. Secondary poisoning is also not uncommon. Metaldehyde is highly toxic by inhalation, moderately toxic by ingestion and slightly toxic by dermal

absorption. Ingestion is the most common route of poisoning. WHO classifies metaldehyde as a moderately hazardous pesticide, and the EPA classifies it as a slightly toxic chemical (Toxicity Class II or III). This chemical has a label of "Restricted Use Pesticide" because of its potential short-term and long-term effects on wildlife.

The oral LD<sub>50</sub> of metaldehyde is 500mg/kg body weight in dogs, 207mg/kg in cats, 300–400mg/kg in horses, 400–500mg/kg in cattle, 300mg/kg in sheep, 800mg/kg in goats, 400–500mg/kg in pigs, 700mg/kg in rats, 200mg/kg in mice, 500mg/kg in guinea pigs and 1250mg/kg in rabbits (Knowles, 1991; NLM, 1995; Beasley, 1999; Plumlee, 2001). The toxic dose of metaldehyde in dogs and cats is reported to be 45mg/pound of body weight. A minimum lethal dose of 500mg/kg body weight for chicken and 300mg/kg for ducks has been determined. The 96h LC<sub>50</sub> values of metaldehyde in rainbow trout and bluegill are 62ppb and 10ppb, respectively.

The type and severity of signs and symptoms of metaldehyde poisoning varies depending on the amount involved and the nature of the exposure. The most common cause of metaldehyde poisoning is direct ingestion. Metaldehyde is primarily a neurotoxicant, as it produces CNS depression, convulsions and violent muscular contractions in several mammalian species following acute exposure. Acute metaldehyde poisoning is often encountered in pets, especially dogs and cats. Onset of signs of metaldehyde toxicosis usually appear within 15min to a few hours after ingestion. Clinical signs of maximal severity occur within 1–3h of ingestion. In general, toxicity signs of mild metaldehyde poisoning include anxiety, hyperesthesia, foaming at the mouth, vomiting, abdominal pain, diarrhea and muscle twitching. Signs of severe poisoning include hypotension and tachycardia, panting, nystagmus, mydriasis, dehydration, hyperthermia, respiratory depression, convulsions, continuous tonic seizures, coma, ataxia and death due to respiratory failure. Not every poisoned animal may show all these signs, but high fever with muscle twitching is very common. In dogs, hemorrhagic gastroenteritis, hypersalivation and vomiting are also seen. Clinical signs in cats are similar to those described for dogs, but nystagmus is more prevalent in cats (Beasley, 1999).

It is a common observation that exposed pets either die within a few hours from an early episode of acute toxicity or within a few days from liver, kidney and respiratory failure. Symptoms of metaldehyde poisoning in domesticated and wild mammals include inability to stand, blindness, changes in respiratory rate, dehydration, excessive sweating and salivation, seizures and sudden death (Grant and Schuman, 1993).

In cattle, sheep and horses, mild poisoning is evidenced by hypersalivation, ataxia and hyperpnea. In severe poisoning, symptoms include convulsions, sweating, tachycardia and muscle spasms. Death occurs within the first 48 hours of accidental ingestion, and is

attributable to respiratory failure (Von Burg and Stout, 1991). Pathological lesions can be found in the liver, kidney, lungs and GI tract.

Metaldehyde-related poisonings and deaths have been reported in avian species (Baker, 1967; Reece *et al.*, 1985; Andreassen, 1993). A minimal lethal dose of 500mg/kg body weight for chickens and 300mg/kg body weight for ducks has been determined. The clinical signs in avian species include excitability, incoordination, tremors, muscle spasms, torticollis, diarrhea and difficult or rapid breathing. Death of geese and ducks has been observed following accidental exposure. Metaldehyde is considered practically nontoxic to aquatic organisms.

In practical situations, chronic toxicity is very rare. Usually, dosages which are not toxic when given singly do not cause illness when repeated (Dreisbach, 1987; Knowles, 1991). In a 2-year chronic toxicity study (three-generation reproductive studies) in rats, changes in liver enzyme activity and increased liver and ovary weight were found at dietary doses of about 12.5mg/kg/day. Fifty percent of female rats given this dose showed paralysis. At higher doses, metaldehyde is known to adversely affect reproduction and the survival rate of offspring (Sax, 1984). Pregnancy exacerbates the toxicity of metaldehyde.

From experimental studies, there is no evidence that metaldehyde is teratogenic (Verschuren *et al.*, 1975), mutagenic (Quinta and Martire, 1981) or carcinogenic (Verschuren *et al.*, 1975).

## Diagnosis

Diagnosis of metaldehyde poisoning can be established based on a history of exposure and clinical signs. The presence of a blue- or green-colored substance or pieces of metaldehyde bait in the vomitus or GI content can be indicative of metaldehyde exposure. The odor of acetaldehyde in the stomach content is suggestive of metaldehyde exposure. Confirmation and quantitation can be done by determining the metaldehyde/acetaldehyde residue in vomitus or GI tract content, blood or urine (Wardall and Bailey, 1976; Smith, 1987; Keller *et al.*, 1991; Jones and Charlton, 1999; Saito *et al.*, 2008). Laboratory analysis may reveal a mixed metabolic acidosis and respiratory alkalosis. Although pathological findings are nonspecific, lesions can be found in the liver, kidney, lungs and GI tract. Prognosis is usually favorable depending on the amount of metaldehyde ingested and timely treatment.

## TREATMENT

There is no specific antidotal treatment for metaldehyde poisoning. Therefore, treatment rests with supportive



and symptomatic measures that usually consist of: (1) removal of remaining metaldehyde from the GI tract, (2) prevention of absorption by activated charcoal, (3) control of the clinical signs, especially seizures, (4) re-establishing ventilation and administering oxygen and (5) restoring fluid and electrolyte balance to correct acidosis. Administration of 2–5% sodium bicarbonate solution can reduce the conversion of metaldehyde to acetaldehyde.

In the case of dogs, if the patient is asymptomatic, immediately induce vomiting by syrup of ipecac (one teaspoon) or a mixture of hydrogen peroxide and water (one tablespoon), followed by gastric lavage using milk or water. The milk helps decrease further absorption of metaldehyde. Simple osmotic diuresis is helpful. Intravenous fluids are recommended. Administration of activated charcoal (1–4 g/kg, PO) is found to be beneficial, as it prevents further absorption of metaldehyde from the intestines. If necessary, charcoal treatment at half the original dose can be repeated at 6–8 h intervals. Charcoal treatment has been found to reduce absorption of metaldehyde by more than 45% (Shintani *et al.*, 1999). Cathartics can also be used with activated charcoal to assist in removing metaldehyde from the intestinal tract. Gastric lavage and cathartics are found to be an effective treatment for up to 12–24 hours after metaldehyde ingestion. Muscle twitching and spasms in dogs and cats can be controlled by methocarbamol (55–220 mg/kg, IV). Seizures can be treated by using diazepam (0.5–2 mg/kg, IV; repeat if necessary) or sodium phenobarbital (30 mg/kg, IV). Urinary acidosis can be corrected by the administration of sodium bicarbonate. In an experimental study conducted on mice, administration of diazepam (10 mg/kg, IP) or clonidine HCl (0.5 mg/kg, IP) decreased the toxicity of metaldehyde (100 mg/kg, PO). Diazepam at a low dose (0.5 mg/kg body weight) was without any protective effect.

If dogs are poisoned by ingesting a snail bait having metaldehyde along with the carbamate methiocarb, pentobarbitone and atropine should be administered, in addition to other supportive measures (Firth, 1992a, b). In the case of pigs, barbiturates can be used to anesthetize poisoned animals for a period of 6–12 h, so as to allow excretion of metaldehyde and its metabolites.

## CONCLUSIONS

Most often, metaldehyde poisoning is acute in nature and results from accidental or malicious activity. Poisoning is characterized by neurological signs, such as violent convulsions and seizures. Without timely and aggressive treatment, death ensues due to respiratory failure. Since no specific antidote is available, treatment relies upon supportive and symptomatic therapies.

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## Toxicity of herbicides

P.K. Gupta

### INTRODUCTION

Herbicides are phytotoxic chemicals used for destroying various weeds or inhibiting their growth. They have variable degrees of specificity. Some, such as paraquat, kill all green plants, whereas phenoxy compounds are specific for certain groups of plants. The worldwide consumption of herbicides is almost 48% of the total pesticide usage. The consumption of herbicides in developing countries is low because weed control is mainly done by hand weeding (Gupta, 2004). Early chemicals used as herbicides include sulfuric acid, sodium chlorate, arsenic trioxide, sodium arsenate and petroleum oils. Iron and copper sulfate or sodium borate were generally difficult to handle and/or toxic, relatively nonspecific, or phytotoxic to the crop as well as the unwanted plant life if not applied at exactly the proper time. The biochemical differences in plants make it possible to design herbicides that have selective toxicity potential against various species of plants. In approximately the past two decades, the herbicides have represented the most rapidly growing section of the pesticide industry due in part to (1) movement into monoculture practices and (2) mechanization of agricultural practices because of increased labor costs. The result has been a plethora of chemically diverse structures rivaling the innovative chemistry so as to develop synthetic organic herbicides and biopesticides that are quite selective for specific plants and have low mammalian toxicity. The aim is to protect desirable crops and obtain high yields by selectively eliminating unwanted plant species, thereby reducing the competition for nutrients (Gupta, 2006a).

Most of the animal/human health problems that result from exposure to herbicides are due to their improper use or careless disposal of containers (Gupta, 2010a). Very few problems occur when these chemicals are used properly. However, there is increased concern about the effects of herbicides on animal health because of runoff from agricultural applications and entrance into drinking water supply (Gupta, 1986, 1988).

### BACKGROUND

The first discovery in the field of selective weed control was the introduction of 2,4-dinitro-*o*-cresol (DNOC) in France in 1933. This is very toxic to mammals and can cause bilateral cataract in humans. In 1934, phenoxy herbicides were developed and 2,4-dichlorophenoxyacetic acid (2,4-D) was introduced (Gupta, 2010b). During World War II, considerable effort was directed toward the development of effective, broad-spectrum herbicides with a view to both increasing food production and finding potential chemical warfare agents (Gupta, 1989). One chemical class of phenoxy derivatives including the acids, salts, amines and esters represents the first commercially available products evolving from this research in 1946. Some other herbicides used from this class include 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB), 2-(2,4-dichlorophenoxy propionic acid) (dichlorprop), 2-(2-methyl-4-chlorophenoxy) propionic acid (MCPP or mecoprop) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (Kennepohl *et al.*, 2010). This class of herbicides

has been in continuous, extensive and uninterrupted use since 1947 and is the most widely used family of herbicides. Another chemical class of herbicides deserving particular attention is the bipyridyl group, especially paraquat and diquat. Weidel and Russo first described the structure of paraquat in 1882. In 1933, Michaelis and Hill discovered its redox properties and called the compound methyl viologen. Its herbicidal properties were discovered by ICI in 1955, and it became commercially available in 1962 (Smith, 1997; Lock and Wilks, 2010).

Ureas and thioureas are a group of herbicides used for general weed control in agricultural and non-agricultural practices. The first urea herbicide, *N,N*-dimethyl-*N'*-(4-chlorophenyl)-urea, was introduced in 1952 by DuPont under the common name of monuron. In subsequent years, many more derivatives of this class of compounds have been marketed (Liu, 2010).

Protopyrinogen oxidase (Protox)-inhibiting herbicides have been used since the 1960s and currently represent a relatively large and growing segment of the herbicide market. Nitrofen was the first Protox-inhibiting herbicide to be introduced for commercial use in 1964. This diphenyl ether (DPE) herbicide was eventually recognized as a relatively weak inhibitor of Protox, but it was a lead compound of an entire class of structurally related herbicides that were much more active. Subsequently, several DPE herbicides have been successfully commercialized (Nandihalli *et al.*, 1992; Anderson *et al.*, 1994).

Substituted aniline, an alachlor herbicide, was registered and introduced in 1967 for the pre-plant or pre-emergent control of a broad spectrum of grass, sedge and broadleaf weeds (Heydens *et al.*, 2010). Subsequently, inhibitors of aromatic acid biosynthesis herbicides (organic phosphorus) such as glyphosate, broad-spectrum, nonselective, post-emergent, systemic herbicide with activity on essentially all annual and perennial plants have been developed. Monsanto discovered the herbicidal properties of glyphosate in 1970, and the first commercial formulation was introduced in 1974 under the Roundup brand name. Other triazine and triazole herbicides have been extensively used in agriculture in the United States and other areas of the world for more than 50 years. The triazines inhibit photosynthesis by blocking photosynthetic electron transport (Gysin and Knuesli, 1960; Steven and Summer, 1991; Breckenridge *et al.*, 2010). Dicamba, which was first registered in the United States in 1967, is another organic (benzoic) acid herbicide that acts by mimicking the effects of auxins (i.e., natural plant growth hormones), causing enhanced but uncontrolled growth rates, alterations in plant function homeostasis and death (Harp, 2010). Another class of synthetic chemical compounds called the imidazolinone herbicides was discovered in the 1970s, with the first U.S. patent awarded in 1980 for imazamethabenz-methyl. New families of herbicides

introduced since the 1970s account for increasing shares of use and include bipyridyl (paraquat), bentazon, fenaxalactogen, oxyfluorfen, clomazone, clorpyralid, fluazifop and norfluorazon. Today, the use of newer compounds that have low toxicity is quite common (Osteen and Padgitt, 2002).

## TOXICOKINETICS

Toxicokinetics is associated with the absorption, distribution, metabolism and excretion of drugs and xenobiotics. Toxicokinetics studies provide important data on the amount of toxicant delivered to a target as well as species-, age- and gender-specific metabolism. Animals are exposed to herbicides of different chemical classes. They may be ingested or absorbed through the skin or the respiratory system. Different factors regulate their absorption, distribution, metabolism and excretion (Gupta, 2010b). In general, liver is the primary site for biotransformation and may include activation as well as detoxification reactions through the cytochrome P450-dependent monooxygenase system, the flavin-containing monooxygenase, esterases and a variety of transferases, most notably the glutathione (GSH) *S*-transferases (Hodgson and Meyer, 1997).

2,4-D is the most extensively studied phenoxy acid derivative herbicide. Absorption of 2,4-D occurs rapidly from the gastrointestinal (GI) tract, and peak levels are reached in 10 min to 24 h depending on species, dose and chemical form. Following oral exposure to 2,4-D, plasma half-lives range from 3.5 to 18 h. Dermal absorption was reported to occur rapidly but was usually less than 6%. The compound is protein bound *in vivo* and is rapidly distributed to the liver, kidneys, lung and brain. 2,4-D has also been reported to cross the placental barrier in laboratory animals and pigs. 2,4-D is not metabolized to reactive intermediates, does not accumulate in tissues and is excreted predominantly as the parent compound in urine. However, the rate of excretion via urine is inversely proportional to dose. 2,4-D has been detected in the milk of lactating rats dosed with 2,4-D. The salts and esters of 2,4-D undergo acid and/or enzymatic hydrolysis to form 2,4-D acid, and small amounts may be conjugated with glycine or taurine. Excretion can be markedly enhanced by ion trapping using alkaline agents because most of these herbicides are organic acids (Erne, 1966a,b; Pelletier *et al.*, 1989; Kennepohl *et al.*, 2010).

Another organic acid herbicide, dicamba, is rapidly and nonselectively distributed to most of the organs; however, dermal absorption is minimal. Ninety percent of excretion is through urine, and a small amount is excreted in feces. Dicamba is mostly unmetabolized

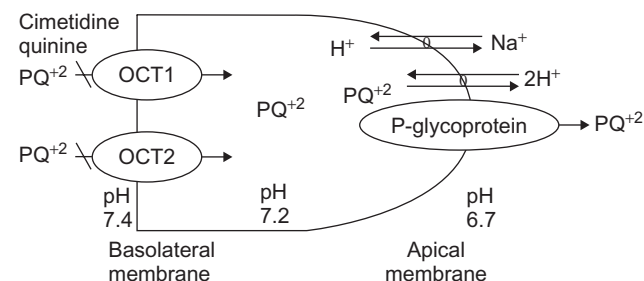


but may be conjugated with glucuronic acid or glycine. Elimination occurs rapidly, and there is no evidence of bioaccumulation in the mammalian system (Harp, 2010).

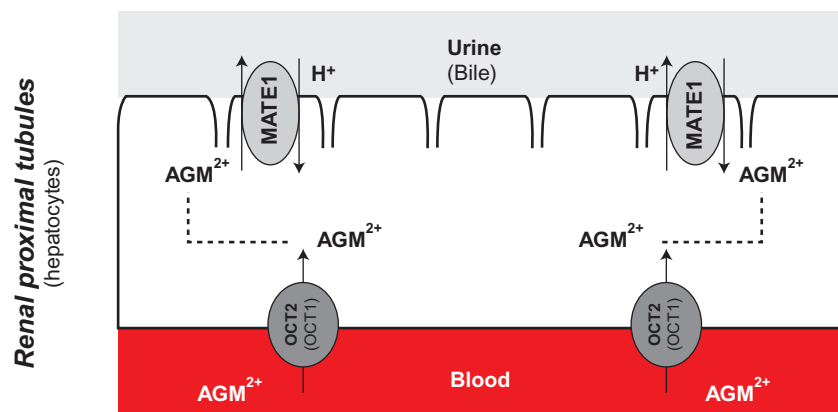
Bipyridyl derivative paraquat is rapidly but incompletely absorbed from the GI tract of laboratory animals and humans, with plasma concentration of 30–90 min, and it is poorly absorbed through contact with skin. It has been reported that dogs absorb more paraquat than do rats, resulting in greater susceptibility of dogs toward paraquat toxicity (Lock and Wilks, 2010). Distribution studies show higher radioactivity in type I and type II epithelial cells and the Clara cells of the rodent and human lungs, which are the major target cells for paraquat toxicity (Smith, 1997). Radioactivity has also been detected in choroid plexus, muscle and melanin in addition to excretory pathways such as the proximal tubules of the kidney, urine, liver, gallbladder and intestinal contents of the mouse, indicating some biliary excretion (Waddell and Marlowe, 1980). Paraquat is taken up into the brain via the neural amino acid transporter; thus, it may be a factor in the etiology of Parkinson's disease. The amount of paraquat excretion in feces corresponds to 60–70% of the ingested dose

(Van Dijk *et al.*, 1975). Paraquat is very poorly metabolized, and bulk is excreted unchanged in the urine and feces. The transport mechanism for organic cations in renal proximal tubular cells is not fully understood; however, two membrane proteins, organic cation transporter 1 (OCT1) and organic cation transporter 2 (OCT2), have been isolated from rat kidney. OCT1, located at the basolateral membrane, transports tetraethylammonium, and this can be inhibited by other organic cations such as quinine. OCT2 stimulates the uptake of tetraethylammonium, and this can be markedly inhibited by cimetidine. The transport of paraquat can be blocked by the addition of the divalent cation quinine, cimetidine and, to a lesser extent, tetraethylammonium, suggesting that paraquat may be transported by both transport systems, an electro neutral organic cation/ $H^+$  exchange and P-glycoprotein (Chan *et al.*, 1998). It was found that the hMATE1-mediated transport of agmatine was inhibited by paraquat, which indicates the involvement of MATE-1 in paraquat renal transport (Winter *et al.*, 2011). It is clear that paraquat can enter a renal cell via OCT2 and, to a lesser extent, OCT1 and then be transported out of the cell by MATE-1 (multidrug and toxin extrusion). However, whether MATE-2k can transport paraquat is not known (Chan *et al.*, 1998; Lock and Wilks, 2010; Winter *et al.*, 2011). A schematic representation of the proposed transport systems for paraquat across renal tubular cells is shown in Figures 54.1 and 54.2.

Unlike paraquat, diquat does not accumulate in the lungs; however, it is observed in liver, kidney, plasma and adrenal gland. Diquat does not enter the brain (Rose *et al.*, 1976). Following oral administration, 90–98% of the dose is eliminated via the urine (Daniel and Gage, 1966). Metabolism studies indicate some unidentified metabolites of diquat in the urine of rabbits and guinea pigs. In rat, diquat monopyridone has been identified in the feces, at approximately 5% of an oral dose, whereas diquat-dipyridone has been detected in urine. These results indicate that diquat is probably metabolized by GI bacteria (JMPR, 1993).



**FIGURE 54.1** Schematic representation of the proposed transport systems for paraquat across renal tubular cells. The transporters are OCT1 and OCT2 at the basolateral membrane and P-glycoprotein and the cation/ $H^+$  exchange system at the brush border membrane. Reproduced with permission from Chan *et al.* (1998).



**FIGURE 54.2** Proposed mechanism of agmatine transport in tissues (i.e., kidney and liver) widely recognized to express OCT1, OCT2 and MATE1. Organic cation transporter (OCT) 1 and 2 mediate the facilitated influx transport of organic cations at the basolateral membrane of hepatocytes and renal proximal tubule cells, respectively. The multidrug and toxic compound extrusion (MATE) transporter 1, an  $H^+$ /cation antiporter, is critical in the efflux elimination of various organic cations from the brush border and canalicular membrane of the kidney and liver, respectively. (Descriptions in parentheses refer to equivalent structures in the liver.) Reproduced with permission from Winter *et al.* (2011)

Ureas and thioureas such as diuron are readily absorbed through the GI tract in rats and dogs and are mainly metabolized by dealkalization of the urea methyl groups. Hydrolysis of diuron to 3,4-dichloroaniline and oxidation to 3,4-dichlorophenol as well as dehydroxylation at carbon 2 and/or carbon 6 of the benzene ring have also been reported. The predominant metabolite of diuron in urine is *N*-(3,4-dichlorophenyl)-urea. Diuron is partially excreted unchanged in feces and urine. The storage of diuron does not occur in tissues (Boehme and Ernst, 1965; Hodge *et al.*, 1967; Liu, 2010).

Organophosphorus herbicides such as glyphosate and glufosinate are poorly absorbed both orally and via the dermal route. There is rapid elimination, and these are not biotransformed and do not accumulate in tissues. More than 70% of an orally administered dose of glyphosate is rapidly eliminated through feces and 20% through urine. The main metabolite of glyphosate is aminomethylphosphonic acid (AMPA); AMPA is of no greater toxicological concern than its parent compound (JMPR, 2004).

The proton class of oxidase inhibitor herbicides is either not readily absorbed or is rapidly degraded by metabolism and/or excreted. The mammalian metabolites are similar to photochemical degradation products. In mammals, there are remarkable species differences in the levels of porphyrin accumulation resulting from exposure to Protox inhibitors. There is no bioaccumulation risk in animals. Metabolism of Protox inhibitors has been studied in a number of species, including rats, rabbits, goats, sheep, cattle and chicken. In general, the metabolic degradation of these compounds by animals includes nitroreduction; deesterification; and conjugation to GSH, cysteine and carbohydrates. Most of the metabolites are excreted in urine, with small amounts excreted in feces and milk. In chickens, approximately 95% of the metabolites are eliminated in excreta, with small amounts (0.09%) eliminated in the eggs (Hunt *et al.*, 1977; Leung *et al.*, 1991).

The carboxyester group of the triazolinone herbicide carfentrazone ethyl is initially metabolized to a carboxylic acid group. Other metabolites identified in rats and lactating goats include hydroxymethylpropionic acid and cinnamic acid derivatives, which are further metabolized to yield a benzoic acid derivative (Aizawa and Brown, 1999).

Substituted anilines are well absorbed in rats orally. The dermal penetration in monkeys is relatively slow. The metabolism of alachlor in rats is complex due to extensive biliary excretion, intestinal microbial metabolism and enterohepatic circulation of metabolites. The main routes of excretion are urine and feces, and nearly 90% of the dose is eliminated in 10 days. The metabolism in rats and mice is similar; however, there are significant quantitative differences between the two species.

These herbicides undergo O-demethylation with the release of formaldehyde; however, the relationship between metabolism-mediated formaldehyde release and nasal tumors induced has not been established. In contrast, alachlor is metabolized in monkeys to a limited number of GSH and glucuronide conjugates, which are excreted primarily via kidney. Excretion in monkeys is more rapid than in rodents, with approximately 90% being excreted in the urine within 48 h. Alachlor metabolites undergo biliary excretion and hepatic circulation in rodents, whereas biliary excretion is limited in monkeys. In rats, acetochlor is rapidly metabolized to several polar metabolites, and more than 95% is quickly excreted in urine and feces. The metabolites are the result of the mercapturic acid pathway formed by initial GSH conjugation. As in the case of alachlor, acetochlor, butachlor and propachlor also undergo glucuronide/glutathione conjugate and enterohepatic circulation leading to tertiary amide methylsulfide metabolite, which further undergoes metabolism in liver and nasal tissue to form the putative carcinogen diethyl quinoneimine (DEIQ) in rats (Millburn, 1975; Feng *et al.*, 1990; Heydens *et al.*, 2010).

Dimethenamid, an amide derivative, is slowly but well absorbed after oral administration (90% in rats) and is extensively metabolized in rats. The maximum concentration in blood is not achieved until approximately 72 h. Excretion is primarily via bile. By 168 h after treatment, an average of 90% of the administered dose is eliminated. In rats, the concentration of radioactivity in blood decreases more slowly than in other tissues and is associated with specific binding to globin (not in humans). Metabolism is primarily via the GSH conjugation pathway, but racemic dimethenamid is also metabolized by cytochrome P450 enzymes via reductive dechlorination, oxidation, hydroxylation, O-demethylation and cyclization pathways, as well as conjugation with glucuronic acid. Unchanged dimethenamid in excreta accounts for only 1 or 2% of the administered dose; more than 40 metabolites have been detected (JMPR, 2005).

In rats, the triazolopyrimidine compounds are rapidly absorbed and urinary elimination is rapid, with half-lives ranging from 6 to 12 h. Excretion is mainly through urine, and small amounts are excreted in feces. The only metabolite present is the 4-OH phenyl derivative and/or oxidation product. In metosulam toxicity, demethylation of the 3-methyl moiety of the phenyl ring and of the 3-methoxy moiety of the pyridine ring and other conjugation products of the parent material have been observed. Other minor metabolites include hydroxylated products of the pyridine ring, although the position of hydroxylation has not been identified. Due to rapid elimination, there is little potential to accumulate in the tissues (Billington *et al.*, 2010).

## MECHANISM OF ACTION

There are a number of biochemical changes or free radical-mediated processes; some may also be produced by other mechanisms that have been used to assess tissue injury. For example, the loss of tissue GSH may reflect alkylation reactions, not oxidation. Furthermore, some free radical-mediated changes that may cause injury are also the result of injury. This is exemplified by the phenomenon of lipid peroxidation that is invoked as a toxic mechanism in many situations but also occurs subsequent to cell death and membrane lysis. In most situations, it is difficult to pinpoint the exact mechanism of action. The mechanism of action of phenoxy derivatives, triazines, triazolopyrimidines, imidazolinones, dinitroaniline and many other classes of herbicides is not precisely known. However, phenoxy compounds are known to depress ribonuclease synthesis, uncouple oxidative phosphorylation and increase the number of hepatic peroxisomes. The relationship of these biochemical changes to clinical effect is not clear. In dogs, these herbicides may directly affect muscle membranes (Sandhu and Brar, 2000). Herbicides such as 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and dicamba act as peroxisome proliferators. Oxadiazinon causes hepatic porphyria in both mice and rats. The phenyl urea herbicides linuron and monuron are rodent liver carcinogens. Chloroacetanilide and metolachlor have shown weak hepatocarcinogenicity in female rats and are non-genotoxic, suggesting a tumor-promoting action. The dinitro compounds markedly stimulate respiration while simultaneously impairing adenosine triphosphate synthesis. The main toxic action is uncoupling of oxidative phosphorylation, converting all cellular energy in the form of heat and causing extreme hyperthermia. In addition, the gut flora in ruminants is able to further reduce the dinitro compounds to diamine metabolites, which are capable of inducing methemoglobinemia.

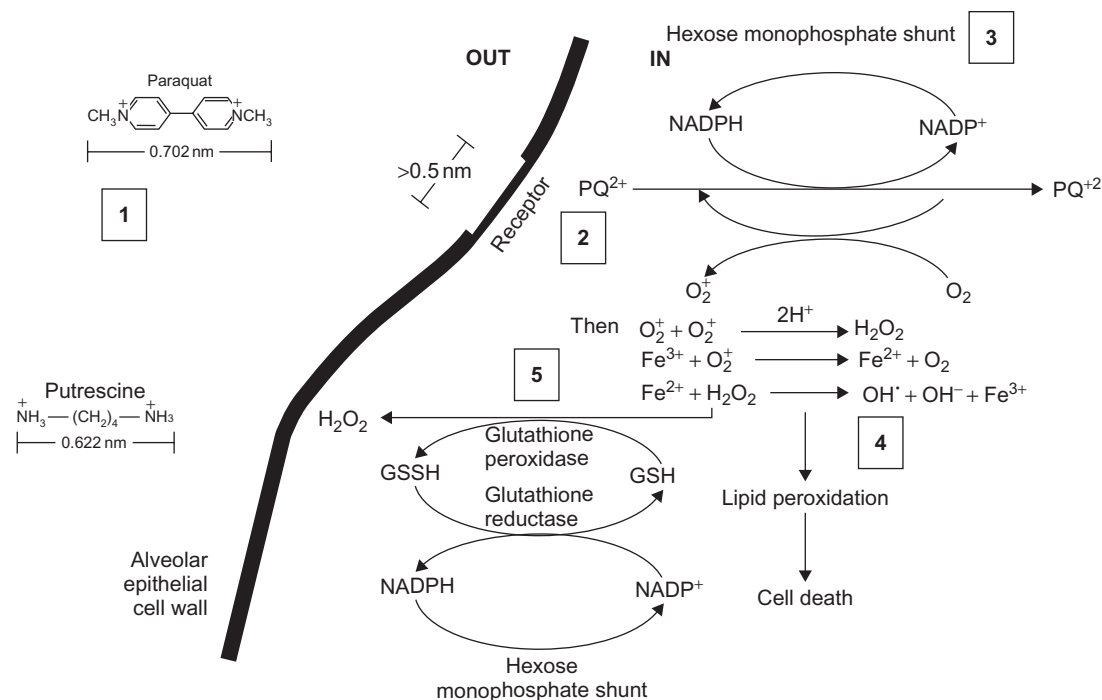
The available information on substituted anilines indicates that there is a nongenotoxic mechanism of action and lack of relevance to humans for the nasal turbinate, stomach and/or thyroid oncogenic effects produced in rats. The data support grouping of alachlor, acetochlor and butachlor with respect to a common mechanism of toxicity for nasal turbinate and thyroid tumors, and grouping of alachlor and butachlor for stomach tumors (Heydens *et al.*, 2010).

The mechanism of action of paraquat and diquat is very similar at the molecular level and involves cyclic reduction–oxidation reactions, which produce reactive oxygen species and depletion of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). However, the critical target organ differs for the two compounds, so the mammalian toxicity is quite different. Although

both herbicides affect kidneys, paraquat is selectively taken up in the lungs. Paraquat causes pulmonary lesions as a result of type I and type II pneumocytosis. The primary event in the mechanism of toxicity within cells is paraquat's ability to undergo a single electron reduction from the cation to form a free radical that is stable in the absence of oxygen. If oxygen is present, a concomitant reduction of oxygen takes place to form superoxide anion ( $O_2^-$ ). Superoxide radical, in turn, is nonenzymatically converted to singlet oxygen, which attacks polyunsaturated lipids associated with cell membranes to form lipid hydroperoxides. Lipid hydroperoxides are normally converted to nontoxic lipid alcohols by the selenium-containing GSH-dependent enzyme, GSH peroxidase. Selenium deficiency, deficiency of GSH, or excess lipid hydroperoxides allows the lipid hydroperoxides to form lipid-free radicals. Lipid hydroperoxides are unstable in the presence of trace amounts of transition metal ions and decompose to free radicals, which in turn cause further peroxidation of polyunsaturated lipid in a process that is slowed by vitamin E. Peroxidation of the membranes could in turn cause cellular dysfunction and hence lead to cell damage or death (Smith, 1997). Genes associated with oxidative stress, redox cycling and apoptosis have been shown to play a key role in the development of lung fibrosis (Tomita *et al.*, 2007; Lock and Wilks, 2010). The neurotoxicity of paraquat is under debate (Lock and Wilks, 2010). A schematic diagram incorporating these elements of the mechanism of paraquat-induced lung toxicity is shown in Figure 54.3.

The mechanism of action of diquat differs somewhat from that of paraquat because it undergoes alternate reduction followed by reoxidation – a process known as redox recycling. Like paraquat, diquat can redox cycle, with the major difference being that diquat can more readily accept an electron than can paraquat (Gage, 1968). The major target organs are the GI tract, the liver and the kidneys. Unlike paraquat, diquat shows no special affinity for the lung and does not appear to involve the same mechanism that selectively concentrates paraquat in the lung (Rose and Smith, 1977).

Glyphosate, a member of the phosphonomethyl amino acid group, selectively inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthetase. The enzyme plays a key role in the biosynthesis of the intermediate, chorismate, which is necessary for the synthesis of the essential amino acids phenylalanine, tyrosine and tryptophan. This aromatic amino acid biosynthesis pathway is found in plants as well as in fungi and bacteria but not in insects, birds, fish, mammals and humans, thus providing a specific selective toxicity to plant species (Franz *et al.*, 1997). Another compound of this group, glufosinate, acts by inhibiting the enzyme glutamine synthetase in animals. Glutamine synthetase in mammals is involved in ammonia homeostasis in many



**FIGURE 54.3** Schematic representation of mechanism of toxicity of paraquat. (1) Structure of paraquat and putrescine; (2) putative accumulation receptor; (3) redox cycling of paraquat utilizing NADPH; (4) formation of hydroxyl radical (OH<sup>•</sup>) leading to lipid peroxidation; and (5) detoxication of H<sub>2</sub>O<sub>2</sub> via GSH reductase peroxidase couple, utilizing NADPH. Reproduced with permission from [Smith \(1997\)](#).

organs and the glutamine–glutamate shunt between  $\gamma$ -aminobutyrate and glutamate in the central nervous system (CNS). However, the enzyme is normally working at a small fraction of its capacity, and considerable inhibition is required in mammals before ammonia levels increase. Hence, this does not lead to a problem in ammonia metabolism; the mammals obviously compensate by using other metabolic pathways ([Hack et al., 1994](#); [JMPR, 2000](#)).

## TOXICITY

More than 200 active ingredients are used as herbicides; however, some are believed to be obsolete or have been discontinued. Of these, several have been evaluated for their toxic potential, and acceptable daily intake has been recommended by the Joint Meeting on Pesticide Residues ([IPCS, 2002](#)). The herbicides may be classified by their chemical structure, but this is not very informative because of overlapping biological effects of a variety of chemical structures. Herbicides may also be classified based on their use, such as how and when the agents are applied. For example, pre-planting herbicides are applied to the soil before a crop is seeded; pre-emergent

herbicides are applied to the soil before the usual time of appearance of the unwanted weeds. Post-emergent herbicides are applied to the soil after the germination of the crops and/or weeds. Classification may also be based on their mechanism of action in plants; their action is referred to as selective (toxic to some species), contact (direct contact), or translocated (through absorption via the soil or through the foliage into the plant xylem and phloem). The World Health Organization (WHO) has recommended classification of pesticides by their acute hazardous nature. Individual products are classified according to the oral or dermal toxicity of technical products and by their physical state. The comparative toxicity of selected herbicides by hazards along with their LD<sub>50</sub> values in rats are summarized in [Table 54.1](#). These values are intended to serve as only a guide because the toxicity of herbicides may be due to the presence of other ingredients (e.g., surfactants and emulsifiers) present in the formulation. The details of guidelines for their hazard evaluation and their classification have been summarized ([IPCS, 2002](#); [Gupta, 2006b](#)). With a few exceptions, most of the newly developed chemicals have a low order of toxicity to mammals. However, there is increasing experimental and anecdotal evidence that exposure to herbicides also affects at least some form of development and/or reproduction in one or more species of animals. A nonexhaustive list of herbicides that



TABLE 54.1 WHO recommended category (hazardous nature) of major chemical classes of technical-grade herbicides with representative examples of acute LD<sub>50</sub> values in rats

Chemical class	Hazardous class	LD <sub>50</sub> (mg/kg BW)	Chemical class	Hazardous class	LD <sub>50</sub> (mg/kg BW)
<b>Phenoxy acid derivatives</b>			Acetochlor	III	2,950
2,4-D	II	375	Butachlor <sup>b</sup>	U	3,300
2,4,5-T	O	–	Metolachlor <sup>b</sup>	III	2,780
Dichlorprop or 2,4-DP	III	800	Propachlor	III	1,500
2,4-DB	III	700	<b>Amides and acetamides</b>		
Dalapon	U	9,330	Bensulide <sup>b</sup>	II	270
MCPB	III	680	Dimethenamid-P	II	429
MCPA	III	700	Propanil	III	1,400 <sup>a</sup>
Mecoprop	III	930	<b>Dinitro compounds</b>		
Mecoprop-P	III	1,050	Binapacryl	O	–
Silvex or fenprop	O	–	DNOC	Ib	25
<b>Bipyridyl derivatives</b>			Dinoterb	Ib	25
Paraquat	II	150	Dinoseb	O	–
Diquat	II	231	<b>Triazolopyrimidines</b>		
<b>Ureas and thioureas (phenyl or substituted ureas)</b>			Cloransulam-methyl	U	>5,000
Chlorbromuron	U	>5,000	Diclosulam	U	>5,000
Chlorotoluron	U	–	Flumetsulam	U	>5,000
Diuron	U	3,400	Metosulam	U	>5,000
Fenuron	U	6,400	<b>Imidazolinones</b>		
Fenuron-TCA	U	4,000	Imazapyr	U	>5,000
Fluometuron	U	>8,000	Imazamethabenzmethy	U	>5,000
Flupyrsulfuron	U	>5,000	Imazethapyr	U	>5,000
Isoproturon	III	1,800	Imazaquin	U	>5,000
Linuron	U	4,000	<b>Benzoic acids</b>		
Metobromuron	U	2,500	Chloramben	U	5,620
Metoxuron	U	>3,200	Dicamba	III	1,707
Monolinuron	U	2,250	Naptalam	U	8,200
Monuron	O	–	<b>Carbamate and thiocarbamate compounds</b>		
Monuron-TCA	O	–	Asulam	U	>4,000
Neburon	U	>10,000	Chlorpropham	U	>5,000
Noruron	O	–	Butylate <sup>b</sup>	U	>4,000
Siduron	U	>7,500	EPTC <sup>b</sup>	II	1,652
Tebuthiuron	III	–	Di-allate	O	–
Thidiazuron	U	>4,000	Pebulate <sup>b</sup>	II	1,120
<b>Organic phosphorus/ phosphonomethyl amino acids or inhibitors of aromatic acid biosynthesis</b>			Terbutol		
Glyphosate	U	4,230	Thiobencarb <sup>b</sup>	II	1,300
Glufosinate	III	1,625	Triallate <sup>b</sup>	III	2,165
<b>Protoporphyrinogen oxidase inhibitors</b>			Vernolate <sup>b</sup>	II	1,780
Nitrofen	O	–	<b>Methyl uracil compounds</b>		
Oxadiazon	U	>8,000	Bromacil	U	5,200
<b>Triazines and triazoles</b>			Terbacil	U	>5,000
<i>Symmetrical triazines</i>			<b>Polycyclic alkanolic acids</b>		
Simazine	U	>5,000	Diclofop	III	565
Atrazine	U	2,000 <sup>a</sup>	Fenoxaprop ethyl	O	–
Propazine	U	5,000	Fenthiaaprop	O	–
Cyanazine	O	–	Fluazifop	O	–
Ametryn	III	1,110	Haloxifop	II	393
Prometryn	U	3,150	<b>Sulfonylureas</b>		
Terbutryn	U	2,400	Chlorsulfuron	U	5,545
Prometon	U	2,980	Sulfometuron	U	>5,000
<i>Asymmetrical triazines</i>			Metsulfuron methyl	U	>5,000
Metribuzin	II	322	Primisulfuron	U	8,200
<b>Substituted anilines</b>			<b>Dinitroanilines</b>		
Alachlor	III	930	Trifluralin	U	>10,000
			Tridiphane	O	–
			<b>Nitriles</b>		
			Ioxynil	II	110
			Bromoxynil	II	190

<sup>a</sup>Variable.<sup>b</sup>Liquid.

Ib, highly hazardous; II, moderately hazardous; III, slightly hazardous; U, unlikely to present acute hazard in normal use; O, obsolete or discontinued for use as herbicide.

Compiled from IPCS (2002) and JMPR (2005).

cause developmental toxicity in experimental animals is presented in Table 54.2. Some herbicides have been associated with birth defects in humans. For example, 2,4-D in combination with 2,4,5-T or dioxin, oryzalin, butiphos, picloram, Silvex (2-(2,4,5-trichlorophenoxy) propionic acid) and TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) are known to cause reproductive problems/malformations in humans. A list of selected herbicides responsible for developmental abnormalities in humans is presented in Table 54.3.

In this chapter, herbicides are classified based on their chemical nature and mechanisms by which they exert biological effects.

**TABLE 54.2** Select herbicides that are known to cause developmental toxicity in experimental animals

Chemical	Malformations
Atrazine	Disruption of ovarian cycle and induced repetitive pseudopregnancy (rats, at high doses)
Buturon	Cleft palate, increased fetal mortality (mice)
Butiphos	Teratogenic (rabbit)
Chloridazon	Malformations
Chlorpropham	Malformations or other developmental toxicity (mice)
Cynazine	Malformations such as cyclopia and diaphragmatic hernia (rabbits); skeletal variations in rats
2,4-D <sup>a</sup> ; 2,4,5-T <sup>a</sup> alone or in combination	Malformations such as cleft palate, hydronephrosis teratogenic (mice, rats)
Dichlorprop	Teratogenic (mice), affect postnatal behavior (rats)
Dinoseb <sup>b</sup>	Multiple defects (mice, rabbits)
Dinoterb	Skeletal malformations (rats), skeletal, jaw, head, and visceral (rabbits)
Linuron	Malformations (rats)
Mecoprop	Malformations (mice only)
Monolinuron	Cleft palate (mice)
MCPA	Teratogenic and embryotoxic (rats); teratogenic (mice)
Prometryn	Head, limb, and tail defects (rat)
Propachlor	Slight teratogenic (rats)
Nitrofen <sup>b</sup>	Malformations (mice, rats, hamsters)
Silvex	Teratogenic (mice)
TCDD <sup>a</sup>	Malformations/teratogenic (fetotoxicity in chickens, rats, mice, rabbits, guinea pigs, hamsters, monkeys)
Tridiphan	Malformations such as cleft palate (mice), skeletal variations (rats)

<sup>a</sup>TCDD is a common contaminant during the manufacturing process of some herbicides, such as 2,4-D and 2,4,5-T.

<sup>b</sup>Obsolete.

Gupta (2011).

## Inorganic herbicides

Substances such as sodium arsenite, arsenic trioxide, sodium chlorate, ammonium sulfamate and borax were formerly used on a large scale. Indeed, the Romans are reputed to have sterilized the soil of Carthage with common salt after the Romans' victory in 146 BC. The disadvantage of such herbicides from an agricultural standpoint is that they are nonselective; thus, their use has declined due to the availability of better and selective organic preparations (Marrs, 2004).

## Phenoxy acid derivatives

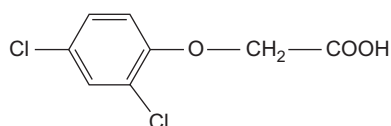
This class of herbicides includes 2,4-D, 2,4,5-T, 2,4-DB, dalapon, dichlorprop or 2,4-DP, mecoprop or MCPP, MCPA and Silvex. Some of the phenoxy derivatives are no longer agents of choice because of the formation of chlorinated dibenzofurans and dibenzodioxins, particularly TCDD, as a consequence of poorly monitored manufacturing practices. Some formulations of 2,4,5-T contain dioxin contaminants that increase the toxicity of technical-grade herbicides and therefore the safe use

**TABLE 54.3** Select herbicides responsible for reproductive problems/malformations in humans

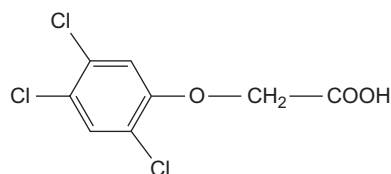
Chemical	Location	Year	Malformations
Butiphos	Russia	1970s	Aggravated parturition, congenital malformations and stillbirths
2,4-D plus 2,4,5-T (Agent Orange)	Vietnam	1960s	Miscarriages, stillbirths and congenital malformations
2,4,5-T and dioxin	New Zealand	1970s	Myelomeningocele
Oryzalin	New York	1970s	Miscarriages, stillbirths and heart defects
Picloram	Brazilian state of Para	1984	Miscarriages and malformed fetuses
Silvex	Arizona	1968–1969	Malformations in pets, domestic animals and wildlife, as well as human reproductive toxicity
TCDD	Times Beach, Missouri	1970s	Miscarriages and birth defects

Gupta (2011).

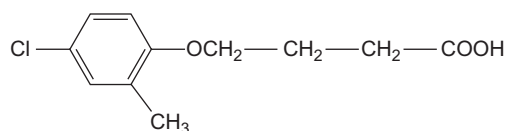
of phenoxy herbicides has been questioned. Published reports have reported the occurrence of three rare forms of cancer (Hodgkin's disease, soft tissue carcinoma and non-Hodgkin's lymphoma) in workers exposed to these herbicides contaminated with dioxins (Kennepohl *et al.*, 2010). However, 2,4-D contains less than the quantitation limits of dioxins set by regulatory agencies (e.g., USEPA). 2,4-D is permitted for use in many countries throughout the world, including the United States and Canada. The structural formulae of selected phenoxy derivatives are given as follows:



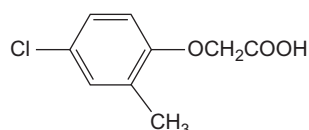
2, 4-dichlorophenoxyacetic acid  
(2, 4-D)



2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T)



2-methyl-4-chlorophenoxybutyric acid (MCPB)



2-methyl-4-chlorophenoxyacetic acid (MCPA)

A tremendous amount of mammalian toxicity data have been collected during the past 60 years. As a group, these are essentially nontoxic, and acute oral/dermal exposure to phenoxy herbicides is slightly to moderately hazardous in normal use (Table 54.1). Dermal irritation in rabbits is considered slight for the acid form of 2,4-D and minimal for the salt and ester forms. Eye irritation in rabbits, on the other hand, is severe for the acid and salt forms, but it is minimal for the ester. The oral LD<sub>50</sub> for phenoxy acid derivatives in dogs is 100–800 mg/kg body weight (BW). The dog is more

TABLE 54.4 TD of phenoxy acid derivatives in animals

	Species	mg/kg BW
Phenoxy acid and its sodium salt		
LD <sub>50</sub>	Chickens	547
	Dogs	100–800
LD	Pigs	500
	Hens	380–765
TD	Pigs	100
	Calves	200
Butyl glycol ester		
TD	Cattle	250 for 3 days
	Sheep	250 for 2 days
Amine salts		
TD	Cattle	250 for 10 days
	Sheep	250 for 10 days
		500 for 7 days

Compiled from Lorgue *et al.* (1996).

sensitive and may develop myotonia, ataxia, posterior weakness, vomiting, bloody diarrhea and metabolic acidosis because of difficulty in the renal elimination of such organic acids (Gehring *et al.*, 1976). Kidney effects consisting of reduced cytoplasmic eosinophilia of the epithelial cells lining and some convoluted tubules have been reported in dog. 2,4-D does not produce any testicular/ovarian damage or induce any abnormal reproductive disorders. However, some of the molecules of this class have been reported to cause teratogenic effects in animals at maternally toxic doses (Table 54.2) and reproductive problems in humans (Table 54.3). The group of compounds neither induces adverse effects in the nervous and immune systems nor has any potential to induce cancer or mutagenicity in laboratory animals. 2,4-D was found to be noncarcinogenic to rats, mice and dogs (Yano *et al.*, 1991a,b; Munro *et al.*, 1992; Kennepohl *et al.*, 2010).

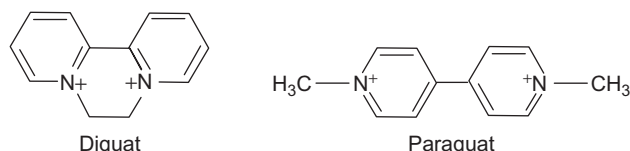
Phenoxy acid derivatives can potentiate the toxic effects of some toxic plants. They may increase the nitrate content of some plant species and can increase the palatability of certain toxic plants (Sudan grass), thus increasing poisoning risks from these plants. Dogs appear to be the most sensitive animals, whereas sheep, cattle and poultry are less sensitive (Table 54.4).

Animals can tolerate repeated oral doses of phenoxy herbicides without showing significant signs of toxicity; suggesting that there is very little cumulative effect on target organs. At high dosages, two types of clinical symptoms – related to the GI tract and the nervous system – have been observed. GI signs include anorexia, rumen atony, diarrhea, ulceration of oral mucosa, bloat and rumen stasis in cattle and vomiting, diarrhea, salivation, etc. in dogs and pigs. Neuromuscular signs include depression and muscular weakness in cattle and ataxia,

posterior weakness (particularly the pelvic limbs) and periodic clonic spasms (at high doses) in dogs. Silvex is unusual for this group because it is very toxic and small doses (2–6 mg/kg BW) may cause ill effects in dogs (Sandhu and Brar, 2000).

### Bipyridyl derivatives

This chemical class of herbicides includes paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride) and diquat (1,1'-ethylene-2,2'-bipyridylium dibromide).



Paraquat is usually formulated as dichloride salt (also known as methyl viologen). The bis(methyl sulfate) salt is no longer commercialized. Paraquat is nonselective and is a fast-acting contact herbicide. This compound is the most toxic of the commonly used herbicides, and the toxicity varies in different animals depending on the formulation and species used. The toxic doses (oral LD<sub>50</sub>) of paraquat and diquat in rats are 150 and 231 mg/kg body weight, respectively, and this class of herbicides is classified as moderately hazardous (Table 54.1). Paraquat is a skin and eye irritant but not a skin sensitizer in animals. Mice are less sensitive than rats to orally administered paraquat, whereas guinea pigs, cats, monkeys and rabbits are more sensitive (Murray and Gibson, 1972; Bus *et al.*, 1976a,b; Nagata *et al.*, 1992; Lorgue *et al.*, 1996; JMPR, 2003; Lock and Wilks, 2010). Cattle and sheep are more sensitive than other species. The comparative toxic oral doses of paraquat and diquat in different species are summarized in Table 54.5.

As indicated previously, paraquat and diquat have somewhat different mechanisms of action. Diquat exerts most of its harmful effects in the GI tract. The major cause of death after exposure to paraquat is lung damage. However, rabbits do not show signs of respiratory distress. Immediate toxic effects include convulsions or depression and incoordination, gastroenteritis and, finally, difficult respiration due to pulmonary edema and alveolar fibrosis (2–7 days). Animals that survive the first few days develop dehydration, pallor or cyanosis, tachycardia, tachypnea, harsh respiratory sounds and emphysema or pneumomediastinum.

Upon long-term exposure, there is progressive pulmonary fibrosis and increased respiratory distress. The

morphological changes seen in animals include degeneration and vacuolization of pneumocytes, damage to type I and type II alveolar epithelial cells, destruction of the epithelial membranes and proliferation of fibrotic cells. The animals die as a consequence of reduced gas exchange and the development of severe hypoxia.

Gross lesions include pulmonary congestion, edema and hemorrhages. Lingual ulcers may be seen. Other findings include failure of lungs to collapse when chest is opened and areas of hemorrhages, fibrosis and atelectasis. Microscopic lesions include necrosis of type I alveolar epithelial cells followed by progressive alveolar and intestinal fibrosis and alveolar emphysema. Renal proximal tubular degeneration and moderate centrilobular hepatic degeneration may also be seen (Smith, 1997). In mice, paraquat did not readily cross the placenta, whereas in rats it readily crossed the placenta, being detected in fetuses within 30 min of an intravenous injection to pregnant rats (Lock and Wilks, 2010). It has neither carcinogenic nor mutagenic potential; however, high doses injected into pregnant rats and mice on various days of gestation may cause significant maternal toxicity but do not produce teratogenic effects (Bus and Gibson, 1975).

Diquat is formulated as dibromide salt and is slightly less toxic to dogs than is paraquat (Table 54.5). After chronic exposure, the major target organs are the GI tract, the liver and the kidneys; however, lungs are not affected (Hayes, 1982). The presence of cataracts in both dogs and rats has been observed. Similar signs of toxicity have been seen in mice, guinea pigs, rabbits, dogs and monkeys. Histopathology injuries to the lining of the stomach and GI tract have been observed, but these are not life-threatening (Clark and Hurst, 1970; Cobb and Grimshaw, 1979). Diquat has no effect on fertility, is not teratogenic

TABLE 54.5 TD of bipyridyl derivatives in animals

	Species	mg/kg BW
Paraquat LD <sub>50</sub>	Dogs	25–50
	Cats	35
	Monkeys	50–70
	Cattle	35–50
	Chickens	110–360
	Sheep	8–10
LD	Pigs	75
Diquat LD <sub>50</sub>	Dogs	100–200
	Cats	35–50
	Cattle	20–40
	Chickens	200–400

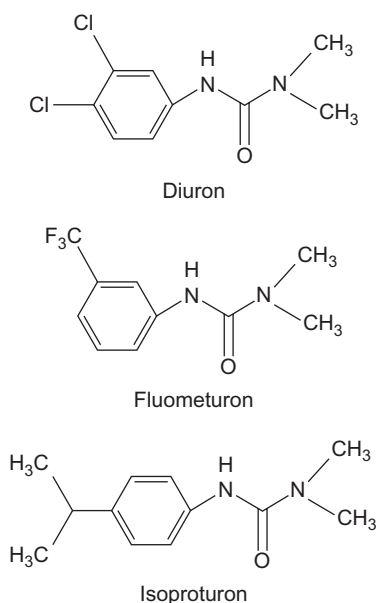
Compiled from Lorgue *et al.* (1996).



and produces fetotoxicity only at doses that are maternally toxic. In a multigeneration study, at high doses, cataracts were observed in rats (FAO/WHO, 1993).

## Ureas and thioureas

The ureas and thioureas (polyureas) are available under different names, such as diuron, fluometuron, isoproturon, linuron, buturon, chlorbromuron, chlortoluron, chloroxuron, difenoxuron, fenuron, methiuron, metobromuron, metoxuron, monuron, neburon, parafluron, siduron, tebuthiuron, tetrafluron and thidiazuron. Of these, diuron and fluometuron are the most commonly used in the United States, whereas isoproturon is mostly used in other countries including India.



In general, polyureas have low acute toxicity and are unlikely to present any hazard in normal use, with the exception of tebuthiuron, which may be slightly hazardous (Table 54.1). The comparative toxic values of polyurea herbicides are summarized in Table 54.6.

Diuron and monuron are potent inducers of hepatic metabolizing enzymes compared to those polyurea herbicides with one or no halogen substitutions (chlortoluron and isoproturon). Male rats are more sensitive than females to the enzyme-inducing activity of diuron, and this can lead to detoxication of EPN and O-demethylation of *p*-nitro anisole. N-demethylation of aminopyrine increases for 1–3 weeks and then returns to normal (Hodgson and Meyer, 1997). Recovery from

TABLE 54.6 TD of ureas and thioureas in animals

	Species	mg/kg BW
Diuron TD	Cattle	100 for 10 days
	Sheep	250 or 100 for 2 days
	Chickens	50 for 10 days
Linuron TD	Dogs	100–200
	Cats	35–50
	Cattle	20–40
	Chickens	200–400
Tebuthiuron <sup>a</sup> TD	Cats	200
	Dogs	50 per day for 3 months (anorexia and weight loss reported)

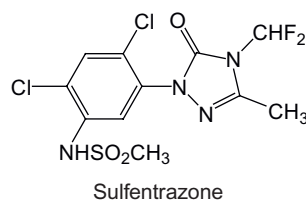
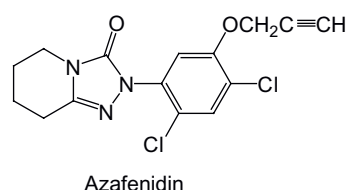
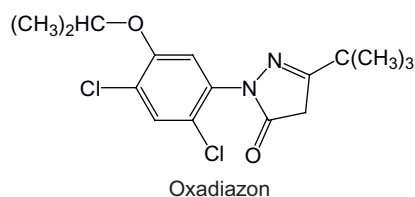
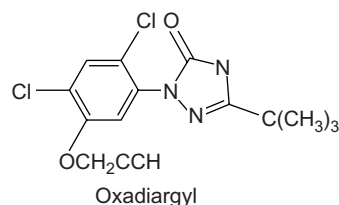
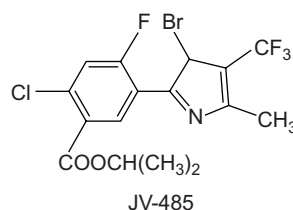
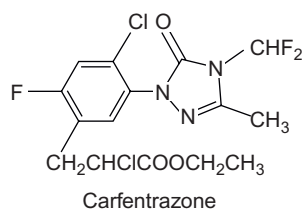
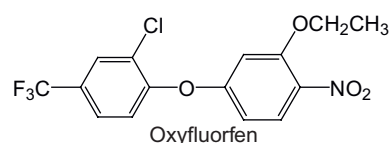
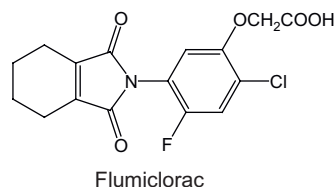
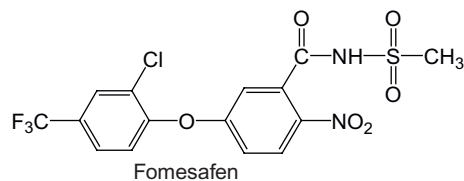
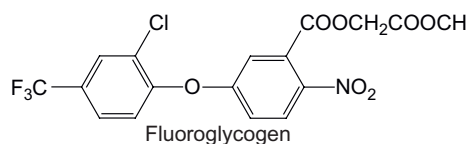
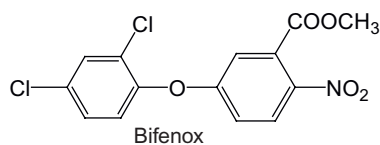
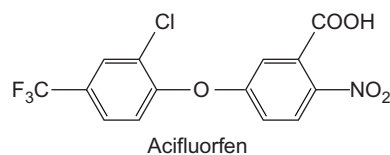
<sup>a</sup>No deaths were reported in dogs, chickens, quail, or ducks at a dosage of 500 mg/kg BW of tebuthiuron.

Compiled from Lorgue *et al.* (1996).

diuron intoxication is quick (within 72 h), and no signs of skin irritation or dermal sensitization have been reported in guinea pigs. After repeated administration, hemoglobin levels and erythrocyte counts are significantly reduced, whereas methemoglobin concentration and white blood cell counts are increased. Dose-dependent relative spleen weight was significantly increased. Increased pigmentation (hemosiderin) in the spleen is seen histopathologically (Liu, 2010). Linuron in sheep causes erythrocytosis and leukocytosis with hypohemoglobinemia and hypoproteinemia, hematuria and ataxia, enteritis, degeneration of the liver and muscular dystrophy. In chickens, it leads to loss of weight, dyspnea, cyanosis and diarrhea. It is nontoxic to fish (Lorgue *et al.*, 1996). Fluometuron is less toxic than diuron. In sheep, depression, salivation, grinding of teeth, chewing movements of the jaws, mydriasis, dyspnea, incoordination of movements and drowsiness are commonly seen. On histopathology, severe congestion of red pulp with corresponding atrophy of the white pulp of the spleen and depletion of the lymphocyte elements have been reported (Mehmood *et al.*, 1995). The acute LD<sub>50</sub> of isoproturon in rats is similar to that of diuron and does not produce any overt signs of toxicity, except at very high doses. A single oral dose of isoproturon in mice may produce some neurotoxic effects at very high doses and may reduce spontaneous and forced locomotor activity (Sarkar and Gupta, 1993a,b).

Polyurea herbicides have been suspected to have some mutagenic effects but do not have carcinogenic potential (Liu, 2010). In general, the compounds do not cause developmental toxicity; however, buturon, linuron and monolinuron are known to cause some teratogenic





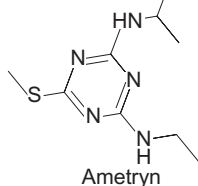
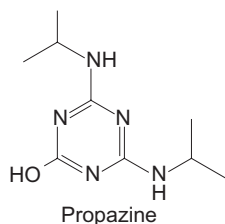
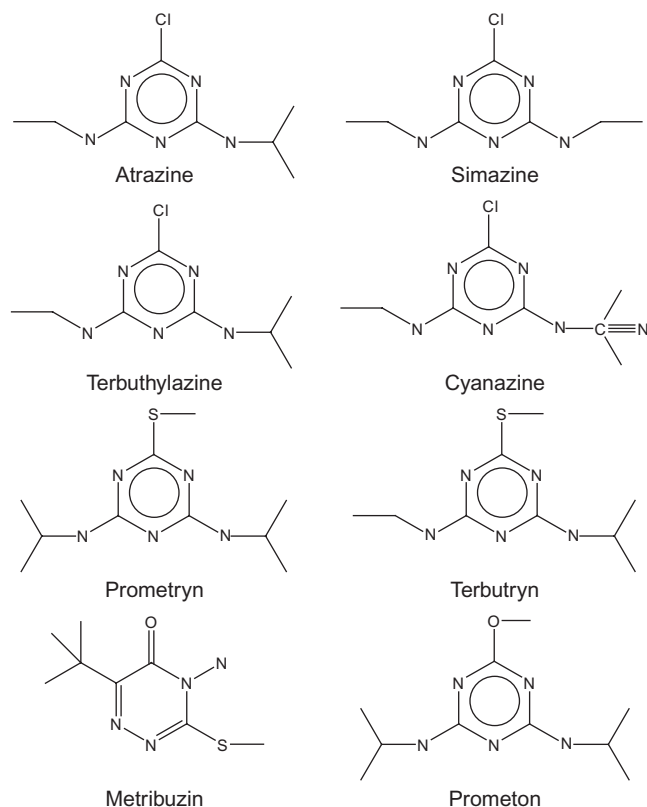
Protox inhibitors have little acute toxicity and are unlikely to pose any acute hazard in normal use (Table 54.1). These compounds increase the porphyrin levels in animals when administered orally, and the porphyrin levels return to normal within a few days. Rats and mice are sensitive and variegate porphyria-like symptoms can be generated in mice with high doses of Protox inhibitors. The majority of these compounds are neither mutagenic nor carcinogenic in nature, and the development toxicity correlates with Protox accumulation. Most Protox inhibitors, except bifenox and oxyfluorfen, are non- to moderately toxic to aquatic wildlife (Dayan and Duke, 2010). It has been reported in rats that

prenatal exposure to sulfentrazone leads to neurodevelopmental effects (de Castro *et al.*, 2007).

### Triazines and triazoles

Triazines and triazoles have been used extensively as selective herbicides for more than 50 years. These herbicides are inhibitors of photosynthesis and include both the asymmetrical and the symmetrical triazines. Examples of symmetrical triazines are chloro-*S*-triazines (atrazine, simazine, propazine, terbuthylazine and cyanazine); the thiomethyl-*S*-triazines (ametryn,

prometryn and terbutryn), and the methoxy-S-triazine (prometon) (Breckenridge *et al.*, 2010). The commonly used asymmetrical triazine is metribuzin. The structures of symmetrical and asymmetrical triazines are given as follows:

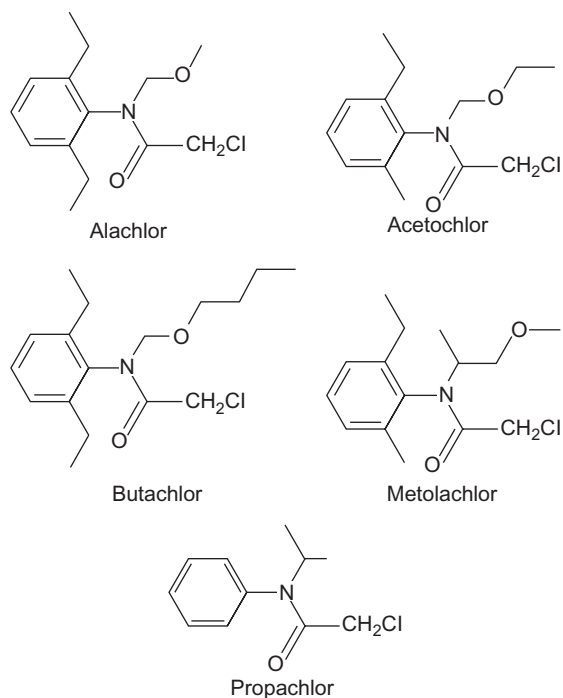


These herbicides have low oral toxicity and are unlikely to pose acute hazards in normal use, except for ametryn and metribuzin, which may be slightly to moderately hazardous (Table 54.1). They are generally neither irritants to the skin or eye nor skin sensitizers. The exceptions are atrazine, which is a skin sensitizer in guinea pigs, and cyanazine, which is toxic by the oral route. However, sensitivity of sheep and cattle to these herbicides is appreciably high. The main symptoms are anorexia, hemotoxia, hypothermia, locomotor disturbances, irritability, tachypnea and hypersensitivity (Sandhu and Brar, 2000). Doses of 500mg/kg of simazine or 30mg/kg atrazine for 30–60 days are

lethal to sheep. Deaths have been reported in sheep and horses grazing triazine-treated pasture 1–7 days after spraying. Cumulative effects are not seen. Metribuzin is slightly more toxic than simazine, but it does not produce any harmful effects in dogs fed at 100ppm in the diet. Simazine is excreted in milk, so it is a public health concern (Susan, 2003). Atrazine is more toxic to rats but comparatively less toxic to sheep and cattle than is simazine. When cultured human cells are exposed to atrazine, splenocytes are damaged but bone marrow cells are not affected. These herbicides are classified as liver microsomal enzyme inducers and are converted to N-dealkylated derivatives. In contrast to simazine, it is not excreted in milk. Triazines seem to have no potential to be mutagenic or to produce carcinogenicity in animals. However, feeding of very high levels of some triazines resulted in mammary tumors in rats. Terbutryn also caused thyroid and liver tumors in female rats (Breckenridge *et al.*, 2010). The exception is cyanazine, which is more acutely toxic, weak mutagenic, and results in developmental toxicity, presumably because of the presence of cyano moiety (Hodgson and Meyer, 1997).

### Substituted anilines

Substituted anilines are used as systemic herbicides. The commonly used herbicides are alachlor, acetochlor, butachlor, metolachlor and propachlor. The structural formulae of selected ones are as follows:





This class of herbicides is slightly hazardous, except butachlor, which is not likely to pose any hazard (Table 54.1). The compounds are nonirritant to eyes, slight to moderate skin irritant, and produce skin sensitization in guinea pigs. Lower doses in rats and dogs do not produce any adverse effects; however, long-term exposure in dogs causes hepatotoxicity and splenic effects. The ocular lesions (progressive uveal degenerative syndrome) produced by alachlor are considered to be unique to the Long-Evans rat because the response has not been observed in other strains of rats, mice, or dogs. At high oral doses, it may lead to maternal and fetal toxicity but may not cause any adverse effect on reproduction. It is neither teratogenic nor produces any microbial genotoxicity. Alachlor has the potential to produce thyroid tumors and adenocarcinomas of the stomach and nasal turbinates of Long-Evans rats and in the lungs (bronchoalveolar) of CD-1 mice at high doses. It is considered to be a human carcinogen (Ahrens, 1994; Monsanto, 1997a,b; Heydens *et al.*, 2010).

Long-term exposure of acetochlor to rats has no adverse effects on reproductive performance. Acetochlor is converted into a rat-specific metabolite that may be related to the nasal and thyroid tumors, thus posing no genetic or carcinogenic hazard to humans (Ashby *et al.*, 1996). Butachlor does not adversely affect reproductive performance or pup survival. It is nongenotoxic. Butachlor induced multiple tumors in SD rats but not in F344 rats or CD-1 mice (Heydens *et al.*, 2010). Metolachlor can increase the incidence of liver tumors in rats and has been classified as a possible human carcinogen (Monsanto, 1991; Wilson and Takei, 1999; Heydens *et al.*, 2010).

Compared to other substituted anilines, propachlor is severely irritating to the eye and slightly irritating to the skin. Propachlor produces skin sensitization in guinea pigs. In rats, high doses of propachlor produce erosion, ulceration and hyperplasia of the gastric mucosa; herniated mucosal glands in the pyloric region of the stomach; hypertrophy; and necrosis of the liver. In dogs, there is poor diet palatability, which results in weight loss and poor consumption of food. Propachlor may produce slight development or adverse reproductive effects (Table 54.2). It is not genotoxic or clastogenic in mammals. However, there is evidence that it produces benign hepatic tumors in male mice (Heydens *et al.*, 2010). The previously discussed data support grouping alachlor, acetochlor and butachlor based on a common mechanism of toxicity for evaluation of risk assessment to humans and animals (Heydens *et al.*, 2010).

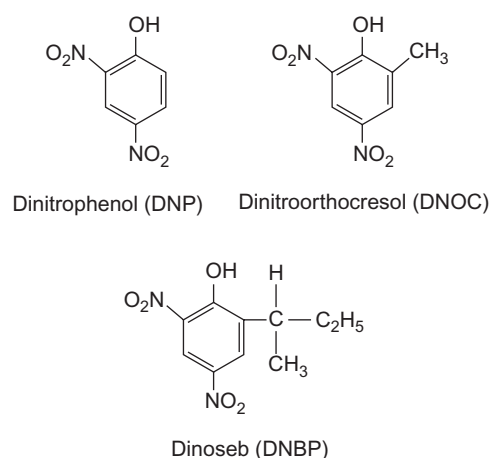
## Amides and acetamides

The commonly used amides and acetamides include bensulide, dimethenamid-P and propanil and are

slightly to moderately hazardous in normal use (Table 54.1). Dimethenamid is a racemic mixture of the M and P stereoisomers, whereas P isomer has useful herbicidal activity. Both substances produce only mild reversible skin and eye irritation and skin sensitization in guinea pigs. Comparison of racemic dimethenamid with dimethenamid-P indicates that there is little difference in their toxicological profiles. The signs of toxicity in mice, rats and dogs are similar, with reduced body weight gain and liver enlargement with induction of liver xenobiotics metabolizing enzyme. There is strong binding to hemoglobin in rats, but this has no relevance to humans. Dimethenamid-P and racemic dimethenamid produce very similar effects in the liver of rats. Both substances produce neither any signs of neurotoxicity nor adverse effects in developmental toxicity or on reproductive performance. Dimethenamid can reduce fetal body weight but is not teratogenic. There is no compound-related mutagenic or carcinogenic potential (JMPR, 2005).

## Dinitrophenol compounds

Several substituted dinitrophenols alone or as salts, such as DNP (2,4-dinitrophenol), DNOC (dinitro-*o*-cresol) and dinoseb (2-(1-methylpropyl)-4,6-dinitro), are used as herbicides. The main source of poisoning in animals is human negligence in removing the preparation if it spills, in disposing of the containers and in preventing animals access to treated fields. The structural formulae of DNP, DNOC and dinoseb are as follows:



In general, the dinitro compounds are not very water-soluble and are highly hazardous to animals (Table 54.1). The oral acute LD<sub>50</sub> of DNOC in mice, guinea pigs, rabbits, hens, dogs, pigs and goats ranges

TABLE 54.7 TD of DNOC and dinoseb in animals

	Species	mg/kg BW
DNOC		
LD <sub>50</sub>	Hens	26
	Dogs	50
	Pigs	50
	Goats	100
TD	Cattle	2–50
	Sheep	20–50
LD	Sheep	25 for 5 days
Dinoseb		
LD <sub>50</sub>	Hens	26
TD	Cattle	25 for 8 days
	Sheep	25 for 10 days

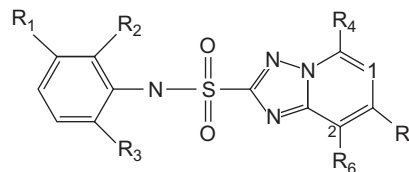
Compiled from Lorgue *et al.* (1996).

from 25 to 100 mg/kg BW. (Table 54.7). In sheep, a dosage of 25 mg/kg/day causes toxicosis in 2–5 days. Clinical signs include fever, dyspnea, acidosis, oliguria, muscular weakness, tachycardia and convulsions followed by coma and death with a rapid onset of rigor mortis. Abortions have been reported in sows. In cattle and ruminants, methemoglobinemia, intravascular hemolysis and hemoproteinemia have been observed. Cataract can occur with chronic dinitrophenol intoxication. Exposure to these compounds may cause yellow staining of skin, conjunctiva, or hair (Lorgue *et al.*, 1996).

## Triazolopyrimidine herbicides

Triazolopyrimidine herbicides include cloransulam-methyl, diclosulam, florasulam, penoxsulam, flumetsulam, metosulam and pyroxsulam. The generic structure of the triazolopyrimidine herbicides connected to a substituted phenyl ring through a sulfonamide bridge is

shown here. The substituents of the various members of this class are summarized in Table 54.8.



Generic structure of the triazolopyrimidine herbicides

The acute oral toxicity of triazolopyrimidine herbicides is very low. On repeated exposure, the primary organs are the kidney (rat and mouse), liver (rat, mouse and dog) and thyroid (rat). Slight decreases in red cell parameters and urine-specific gravity in males, slightly increased cecal and liver weights, slight to moderate hypertrophy of collecting renal tubule epithelial cells and/or slight vacuolization of proximal tubular epithelium, hepatocellular vacuolation, and slight thyroid follicular hypertrophy have been observed (Billington *et al.*, 2010). In most cases, the histopathological changes represent adaptive responses. Analysis of <sup>14</sup>C activity of dog eyes indicated that this organ is a target of toxicity in dogs, exhibiting an affinity for the radiotracer not seen in other species (Timchalk *et al.*, 1996). No adverse effects on neurotoxicity, reproductive performance and mutagenic abnormalities have been observed. The compound has no carcinogenic potential in humans (EPA, 1997a,b).

## Imidazolinones

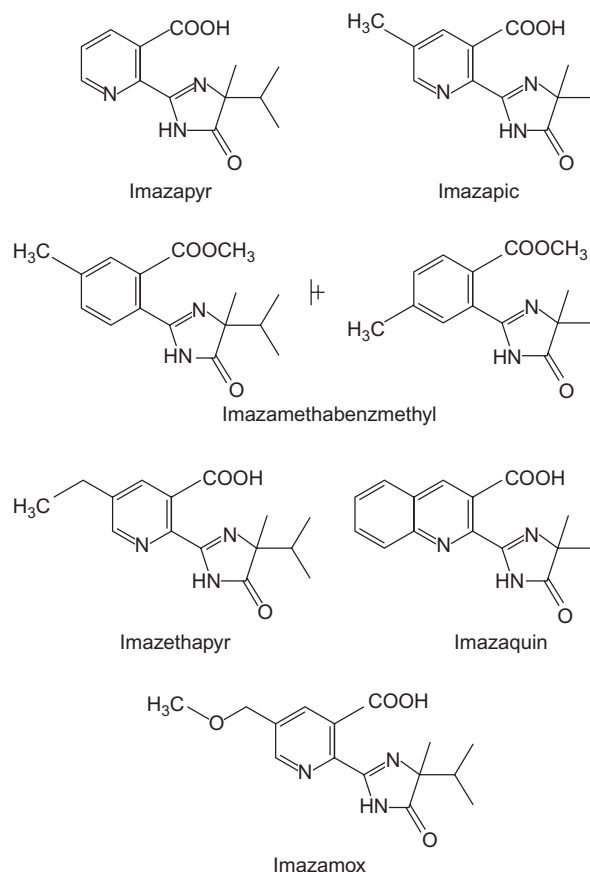
Imidazolinone herbicides include imazapyr, imaza-methabenzmethyl, imazapic, imazethapyr, imazamox and imazaquin. These are selective broad-spectrum

TABLE 54.8 Substituents of triazolopyrimidine sulfonamide herbicides

	1	2	3	4	5	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>
[1,5c] Cloransulam-methyl	N	C	C	NH	SO <sub>2</sub>	H	CO <sub>2</sub> CH <sub>3</sub>	Cl	OCH <sub>2</sub> CH <sub>3</sub>	F	H
Diclosulam	N	C	C	NH	SO <sub>2</sub>	H	Cl	Cl	OCH <sub>2</sub> CH <sub>3</sub>	F	H
Florasulam	N	C	C	NH	SO <sub>2</sub>	H	F	F	OCH <sub>3</sub>	H	F
Penoxsulam a	N	C	C	SO <sub>2</sub>	NH	H	OCH <sub>2</sub> CHF <sub>2</sub>	CF <sub>3</sub>	OCH <sub>3</sub>	H	OCH <sub>3</sub>
[1,5a] Flumetsulam	C	N	C	NH	SO <sub>2</sub>	H	F	F	H	CH <sub>3</sub>	–
Metosulam	C	N	C	NH	SO <sub>2</sub>	CH <sub>3</sub>	Cl	Cl	OCH <sub>3</sub>	OCH <sub>3</sub>	–
Pyroxsulam a	C	N	N	SO <sub>2</sub>	NH	H	CF <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	–

From Billington *et al.* (2010).

herbicides discovered in the 1970s. The structural formulae of this class of herbicides are as follows:

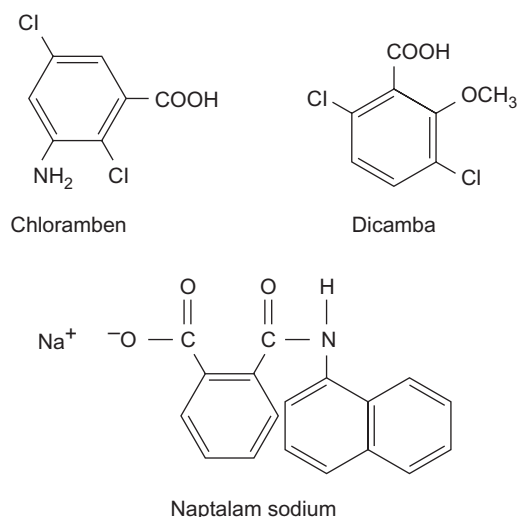


These herbicides are relatively nontoxic (Table 54.1). Results from primary eye irritation studies range from no irritation (imazaquin) to slightly irritating (imazamethabenzmethyl) and moderately irritating (imazapic and imazethapyr), showing complete recovery within 7 days post-dosing. The rabbit primary irritation study with imazapyr showed irreversible irritation. Toxicological effects of imidazolinone herbicides are slight to moderate skeletal myopathy and/or slight anemia in dogs occurring in the 1-year dietary toxicity studies with three structurally similar imidazolinones (imazapic, imazaquin and imazethapyr). There is no evidence of any adverse effect on reproductive performance and on fetal abnormalities in the rat and the rabbit. Neither mutagenicity nor any carcinogenicity has been reported in either of these species (Hess *et al.*, 2010).

### Benzoic acids

The herbicides in this group include chloramben, dicamba and naptalam. These have a low order of

toxicity, and the LD<sub>50</sub> values in rats are summarized in Table 54.1. The structural formulae of selected herbicides are as follows:



In practice, dicamba is often combined with other herbicides and is used to control a wide spectrum of weeds. The signs and lesions are similar to those described for the chlorophenoxy acids. Poisoning after normal use has not been reported in domestic animals. Dicamba either alone or combined with another herbicide induced significant levels of apoptosis in mouse preimplantation embryo assay (Greenlee *et al.*, 2004). It is a skin and eye irritant, and high doses may cause neurobehavioral symptoms in rats and rabbits. The compound did not show any adverse effects in a three-generation study in rats (Harp, 2010).

Dicamba induces peroxisomal enzymes in rat liver and causes transcription up-regulation of the peroxisome proliferator-activated receptor. Long-term exposure to dicamba may induce tumors in rats due to its action as a peroxisome proliferator; however, the implications of these findings are not clear and may require further study (Espandiari *et al.*, 1998). Dicamba-induced oxidative stress-mediated cytogenotoxicity has been demonstrated in an *in vitro* cell model (Gonzalez *et al.*, 2009; Harp, 2010).

### Carbamates, thiocarbamates and dithiocarbamate compounds

The compounds in this category include derivatives of carbamic acid (asulam, barban, chlorpropham, chlorbulfam, karbutilate and phenmedipham), derivatives of thiocarbamic acid (butylate, cycloate, di-allate, EPTC,

TABLE 54.9 TD of asulam and di-allate in animals

	Species	mg/kg BW
Asulam LD <sub>50</sub>	Rabbits	>2000
	Chickens	>2000
	Dogs	>5000
Di-allate LD <sub>50</sub> TD	Dogs	510
	Chickens	150 for 10 days or 250 for 7 days
	Cattle	25 for 5 days or 50 for 3 days
	Sheep	25 for 5 days or 50 for 3 days

Compiled from Lorgue *et al.* (1996).

molinate and tri-allate) and derivatives of dithiocarbamic acid (metham sodium). These herbicides have low to moderate toxicity in rats and do not pose acute hazards (Table 54.1). They are used at low concentrations, and poisoning problems have not been reported. In general, these herbicides do not produce skin or eye irritation. With repeated exposure, there is a possibility of alopecia for some time after ingestion (Lorgue *et al.*, 1996; Hurt *et al.*, 2010). The toxic doses of asulam and di-allate in animals are summarized in Table 54.9.

In ruminants, di-allate results in anorexia, ataxia, muscular contractions, exhaustion, prostration and alopecia in sheep, which is an indication of chronic poisoning. Thiobencarb has induced toxic neuropathies in neonatal and adult laboratory rats. It appears to increase permeability of the blood–brain barrier. The nonspecific lesions include hepatic, renal and pulmonary congestion; enteritis; ascites; and hydrothorax (Susan, 2003).

## Others

Bromacil and terbacil are commonly used methyluracil compounds. These compounds can cause mild toxic signs at levels of 50 mg/kg BW in sheep, 250 mg/kg BW in cattle and 500 mg/kg BW in poultry when given daily for 8–10 days. Signs of toxicity include bloat, incoordination, depression and anorexia. Toxic doses of bromacil can be hazardous, especially for sheep, but no field cases of toxicity have been reported (Table 54.10).

The nitrile herbicides, ioxynil and bromoxynil, may uncouple and/or inhibit oxidative phosphorylation. Ioxynil, presumably due its iodine content, causes enlargement of the thyroid gland in the rat (Marrs, 2004). Members of polycyclic alkanolic acids (diclofop, fenoxaprop, fenthiaprop, fluazifop and haloxyfop) have moderately low toxicity, whereas haloxyfop-methyl is an exception, having high toxicity. They tend to be more toxic if exposure is dermal. The dermal LD<sub>50</sub> of diclofop in rabbits is only 180 mg/kg (Susan, 2003). Some

TABLE 54.10 TD of bromacil in animals

Species	mg/kg BW
Cattle	250
Chickens	500 for 10 days
Sheep	50 for 10 days or 250 for 8 days

Compiled from Lorgue *et al.* (1996).

members of the amide group, such as bensulide and propanil, are used as plant growth regulators, and some of them are more toxic than others (Table 54.1). A lethal dose of bensulide for dogs is 200 mg/kg.

The prominent clinical sign is anorexia; other signs and lesions are not definitive and are similar to those of chlorophenoxy acid poisoning. Hemolysis, methemoglobinemia and immunotoxicity have occurred after experimental exposure to propanil (Lorgue *et al.*, 1996). The toxicity of sulfonylureas (chlorsulfuron, sulfometuron, metsulfuron, chloremuron and kensulfuron) appears to be quite low (Susan, 2003).

A number of substances are used as defoliant in agriculture, including sulfuric acid to destroy potato haulms and two closely related trialkylphosphorothioates (DEF and merphos) to defoliate cotton. A notable feature of the latter is that they produce organophosphate-induced delayed neuropathy in hens (Baron and Johnson, 1964). Chlomequat is used as a growth regulator on fruit trees. The signs of toxicity in experimental animals indicate that it is a partial cholinergic agonist (JMPR, 2000).

## ENDOCRINE DISRUPTION

In both males and females, some herbicides affect reproduction through different mechanisms of action of endocrine disruption; exogenous agents interfere with reproduction and the development process. In males, normal reproductive function involves interaction of the hypothalamic–pituitary–testis axis and the thyroid gland. In females, increased concentrations of xenoestrogens may affect ovarian function through the disruption of feedback mechanisms in the hypothalamus–pituitary–gonadal axis (Flaws and Hirshfield, 1997; Bretveld *et al.*, 2006). Herbicides, like other chemicals, may disrupt all stages of hormonal function of the reproductive system. In females, during pregnancy and, to a greater extent, during lactation, a portion of the maternal body burden of these chemicals is transferred to the offspring. For example, herbicides such as linuron produce hypothyroidism (Gupta, 2011). The herbicide glyphosate in low nontoxic concentrations caused disruption of the



aromatase enzyme in human placental cells *in vitro*. It reduced the aromatase enzyme activity responsible for the synthesis of estrogens (Richard *et al.*, 2005). A study indicated that male reproductive toxicity of glyphosate is due to the inhibition of a StAR protein and an aromatase enzyme, which caused an *in vitro* reduction in testosterone and estradiol synthesis. The study further suggested that commercial formulation of glyphosate (glyphosate-Roundup Transorb, Monsanto) is a potent endocrine disruptor *in vivo* because it caused disturbances in the reproductive development of rats when the exposure was performed during the puberty period (Romano *et al.*, 2010). From various experimental studies, it has been concluded that herbicides can disturb reproduction and development processes of both males and females through endocrine signals in organisms indirectly exposed during prenatal or early postnatal life. Such effects during fetal development may be permanent and irreversible.

According to one estimate, eight herbicides (2,4-D, 2,4,5-T, alachlor, amitrole, atrazine, metribuzin, trifluralin and nitrofen) were identified as endocrine disruptors. Most of these were identified accidentally rather than as a result of an exhaustive screening process (Pocar *et al.*, 2003).

## TREATMENT

The successful management of herbicide poisoning depends on (1) the clinicians' understanding of the mechanism of herbicide toxicity and applying that understanding to the treatment options, (2) accurate diagnosis and assessment of the severity of intoxication, (3) maintenance of vital body functions and adequate clinical monitoring, (4) minimization of further absorption of the compound, and (5) appropriate use of specific treatment. Because the majority of herbicides have relatively low acute and chronic toxicity, no attempt has been made to identify antidotes for intoxication. However, there is a great deal of controversy regarding the purported chronic effects of phenoxy derivatives such as 2,4-D and 2,4,5-T due to the potential for contamination of 2,4,5-T with the highly toxic and unwanted byproduct commonly referred to as dioxin. Treatment is usually symptomatic and supportive. Intravenous fluid should be given to promote diuresis. Similarly, there is no specific treatment for bipyridyl herbicide poisoning. The therapy for bipyridyl poisoning may focus on prevention of absorption from the GI tract. Paraquat binds tightly to diatomaceous clay. Oral administration of adsorbents such as bentonite or Fuller's earth (clay) along with a cathartic such as magnesium sulfate may be

helpful. Toxicity of paraquat is enhanced by selenium/vitamin E deficiency, oxygen and low tissue GSH peroxidase activity. Therefore, vitamin E and selenium with supportive therapy may be useful in the early stages of intoxication. Excretion of bipyridyl compounds may be accelerated by forced diuresis induced by mannitol infusion and furosemide administration. Oxygen therapy and fluid therapy are contraindicated (Clark, 1971; Smith *et al.*, 1974). Dinis-Oliveira and co-workers experimentally found dexamethasone, sodium salicylate and lysine acetylsalicylate to be an effective treatment for paraquat-induced toxicity. Furthermore, they concluded that the antioxidant properties of these agents might be responsible for their effectiveness (Dinis-Oliveira *et al.*, 2006, 2007, 2008, 2009).

An effective antidote for dinitrophenol compounds is not known. Affected animals should be cooled and sedated to help control hyperthermia. Phenothiazine tranquilizers are contraindicated; however, diazepam can be used to calm the animal. Atropine sulfate, aspirin and antipyretics should not be used; rather, physical cooling measures such as cool baths or sponging and keeping the animal in a shaded area are advocated. Intravenous administration of large doses of sodium bicarbonate (in carnivores) solutions, parenteral vitamin A and intense oxygen therapy, where possible, may be useful. If the herbicide is ingested and the animal is alert, emetic should be administered; if the animal is depressed, gastric lavage should be performed. Treatment with activated charcoal should follow. Dextrose-saline infusions in combination with diuretics and tranquilizers (not barbiturates) are very useful. In ruminants, for methemoglobinemia, methylene blue solution and administration of ascorbic acid are useful (Lorgue *et al.*, 1996).

## CONCLUSIONS

Herbicides are routinely used to control noxious plants. Most of these chemicals, particularly the synthetic organic herbicides, are quite selective for specific plants and have low toxicity for mammals; other less selective compounds (e.g., arsenicals, chlorates and dinitrophenols) are more toxic to animals. Most animal health problems including reproduction, which is affected by endocrine disruption, result from exposure to excessive amounts of herbicides because of improper or careless use or disposal of containers. The residue potential for most of these chemicals is low. However, some aspects of herbicidal toxicity, such as the isozyme specificity for metabolism, induction and inhibition in interaction of herbicides with hepatic P450, flavin-containing monooxygenase-dependent oxidations, and phase I and

phase II enzymes, need special attention because these are not clear. A number of other questions remain unanswered, such as; What interactions occur at portals of entry and sites of toxic action? Can this information help us to understand the mechanism of action? In addition, during reproduction, physiological changes occur in virtually every maternal organ/system. These changes may have a profound effect on the toxicokinetics of the chemical. For example, 2,4,5-T is more slowly eliminated in mice as gestation progresses due to increased tissue residence, resulting in potentially higher fetal exposure during the later stages of pregnancy. Indeed, clearance of chemicals from the developing fetus appears to be progressively decreased during gestation in rats. It is therefore a challenge to predict maternal or embryo/fetal exposure at various times during pregnancy. A good understanding of pregnancy-related anatomical and physiological changes in animals and their potential impact on the chemical kinetic is required.

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## Toxicity of fungicides

*P.K. Gupta and Manoj Aggarwal*

### INTRODUCTION

Fungicides are agents that are used to prevent or eradicate fungal infections from plants or seeds. In agriculture, they are used to protect tubers, fruits and vegetables during storage or are applied directly to ornamental plants, trees, field crops, cereals and turf grasses. Numerous substances having widely varying chemical constituents are used as fungicides (Gupta, 1988). Fungicides have been classified according to chemical structures or have been categorized agriculturally and horticulturally according to the mode of action (Ballantyne, 2004). According to the mode of application, fungicides are grouped as foliar, soil and dressing fungicides. Foliar fungicides are applied as liquids or powders to the aerial green parts of plants, producing a protective barrier on the cuticular surface and systemic toxicity in the developing fungus. Soil fungicides are applied as liquids, dry powders, or granules, acting either through the vapor phase or by systemic properties. Dressing fungicides are applied to the postharvest crop as liquids or dry powders to prevent fungal infestation of the crop, particularly if stored under less than optimum conditions of temperature and humidity. Thus, effective fungicides must be protective, curative, or eradivative and should possess the following properties: (1) low toxicity to the plant/animal but high toxicity to the particular fungus; (2) activity *per se* or the ability to convert themselves (by plant or fungal enzymes) into toxic intermediates; (3) the ability to penetrate fungal spores or the developing mycelium to reach the site of action; (4) low ecotoxicity; and (5) the ability to form a protective, tenacious deposit on the plant surface that will be resistant

to weathering by sunlight, rain and wind (Phillips, 2001). With a few exceptions, most of the newly developed chemicals have a low order of toxicity to mammals. Public concern has focused on the positive mutagenicity tests obtained with many fungicides and the predictive possibility of both teratogenic and carcinogenic potential. The quantity of fungicides used on major crops is estimated to have increased 2.3-fold between 1964 and 1997. Use of inorganics (primarily copper compound) and dithiocarbamates has declined since the 1960s, but captan, chlorothalonils and other organic materials account for 90% of fungicide use. Newer groups, such as benzimidazoles, conazoles, dicarboximides and metal organic compounds, account for approximately 10% of fungicide use (Osteen and Padgitt, 2002). In this chapter, fungicides are discussed using a chemical classification system.

### BACKGROUND

The earliest fungicides were inorganic materials such as sulfur, lime, copper and mercury compounds. The use of element sulfur as a fungicide was recommended as early as 1803. It has become an important component of integrated pest management systems because it can be used in "organic farming." There are an increasing number of instances of dermatitis in human farmworkers and diseases in ruminants caused by exposure to high levels of sulfur (Gammon *et al.*, 2010). The mercury-containing fungicides have been responsible for many deaths or permanent neurological disability. Some of the earlier

inorganic metallic fungicides have been withdrawn in many countries because of their toxicity and adverse environmental effects (Ballantyne, 2004). Another compound, hexachlorobenzene (HCB), was extensively used from the 1940s through the 1950s as a fungicidal dressing applied to seed grains as a dry powder. Between 1955 and 1959, an epidemic of poisoning occurred in Turkey and resulted in a syndrome called black sore and caused more than 4000 deaths. Although this agent has largely fallen by the wayside, it is still being used in developing countries. It is a highly toxic compound and can lead to severe skin manifestations including hypersensitivity (Hayes, 1982; Gupta, 2010a). Since that time, many compounds have been developed and used to control fungal diseases in plants, seeds and produce. Carbamic acid derivatives, including ethylenebisdithiocarbamates (EBDCs), are a group of fungicides that have been used widely throughout the world since the 1940s. The important members of this class include mancozeb, maneb, metiram, zineb and nabam. All the members have an EBDC backbone, with different metals associated with the individual compounds. Captan, folpet and captan-fol have been in use for more than 55 years. These compounds belong to the chloroalkylthiodicarbimide class of fungicides due to the presence of chlorine, carbon and sulfur in the side chain. Related compounds associated with this fungicide class are dichlofluanid and tolylfluanid. These two compounds have a fluorine atom substituted for one of the terminal chlorine atoms. Another compound, chlorothalonil, which is a halogenated benzonitrile fungicide, was first registered for use as an agrochemical in the United States in 1966. Chlorothalonil also has wider biocidal applications, including use in paints and lubricant fluids. The benzimidazole fungicides, benomyl and carbendazim, have been in use for more than 40 years, whereas anilinopyrimidines, a new class of fungicides (cyprodinil, mepanipyrim and pyrimethanil), were introduced in 1993 (cyprodinil by Ciba in France) for application on cereal grains (Ollinger *et al.*, 2010).

## TOXICOKINETICS

Toxicokinetic studies provide important data on the amount of toxicant delivered to a target as well as species-specific metabolism. Animals are exposed to fungicides through ingestion or they are absorbed through the skin or the respiratory system. Different factors regulate their absorption, distribution, metabolism and excretion. In general, the liver is the primary site for biotransformation and may include detoxification as well as activation reactions (Gupta, 1986). Some fungicides do not undergo

any metabolism and bind with other active binding sites. The aryl organomercurials methyl- and ethylmercury chloride are poorly excreted and tend to accumulate in muscle, brain and other tissues, whereas the aryl organomercurial phenylmercury is more readily excreted via the kidney and less likely to accumulate in brain and muscles. Similarly, HCB possesses all the properties of chemical stability, slow degradation and biotransformation, environmental persistence and bioaccumulation in adipose tissue and organs containing a high content of lipid membranes (Costa, 2008). The newly introduced class of fungicides are rapidly absorbed, metabolized and excreted and do not accumulate in tissues, but some of them are partially absorbed from the gastrointestinal (GI) tract. For example, absorption of chlorothalonil from the GI tract is on the order of 30–32% of the administered dose. At least 80% of the administered dose is excreted in feces within 96 h. The highest concentrations are observed in the kidneys – approximately 0.1% of the dose. In this case, gut microflora play a role in the disposition and metabolism in rats. Glutathione conjugation plays a central role in the metabolism and subsequent complex metabolic processing of these conjugates, resulting in selective renal uptake and urinary excretion of thiol-derived metabolites. Hepatic glutathione levels are decreased, and renal glutathione levels are elevated. The depletion of hepatic glutathione is considered a direct consequence of glutathione conjugation within the liver utilizing tissue resources. The increase in renal glutathione content is more difficult to explain, but it may be a consequence of urinary excretion of glutathione conjugates (Parsons, 2010).

The captan is rapidly degraded to 1,2,3,6-tetrahydrophthalimide (THPI) and thiophosgene (via thio-carbonyl chloride) in the stomach before reaching the duodenum. THPI has a half-life of 1–4 s, and thiophosgene is detoxified by reaction with cysteine or glutathione and is rapidly excreted. No captan is detected in blood or urine. It is therefore unlikely that these compounds or even thiophosgene would survive long enough to reach systemic targets such as the liver, uterus, or testes. Due to rapid elimination, meat, milk, or eggs from livestock/poultry would be devoid of the parent materials. Humans appear to metabolize captan in a similar manner to other mammals (Krieger and Thongsinthusak, 1993; JMPR, 2004; Gordon, 2010).

Cyprodinil, an anilinopyrimidine class fungicide, is rapidly absorbed from the GI tract into systemic circulation in rats. Approximately 48–68% of the administered dose is excreted in the urine, whereas 29–47% is found in the feces. Total excretions reach 92–97% of the administered dose within 48 h. Cyprodinil is almost completely metabolized. No unchanged parent molecule is found in urine, whereas minor amounts of unchanged cyprodinil are found in feces. Most of the administered cyprodinil

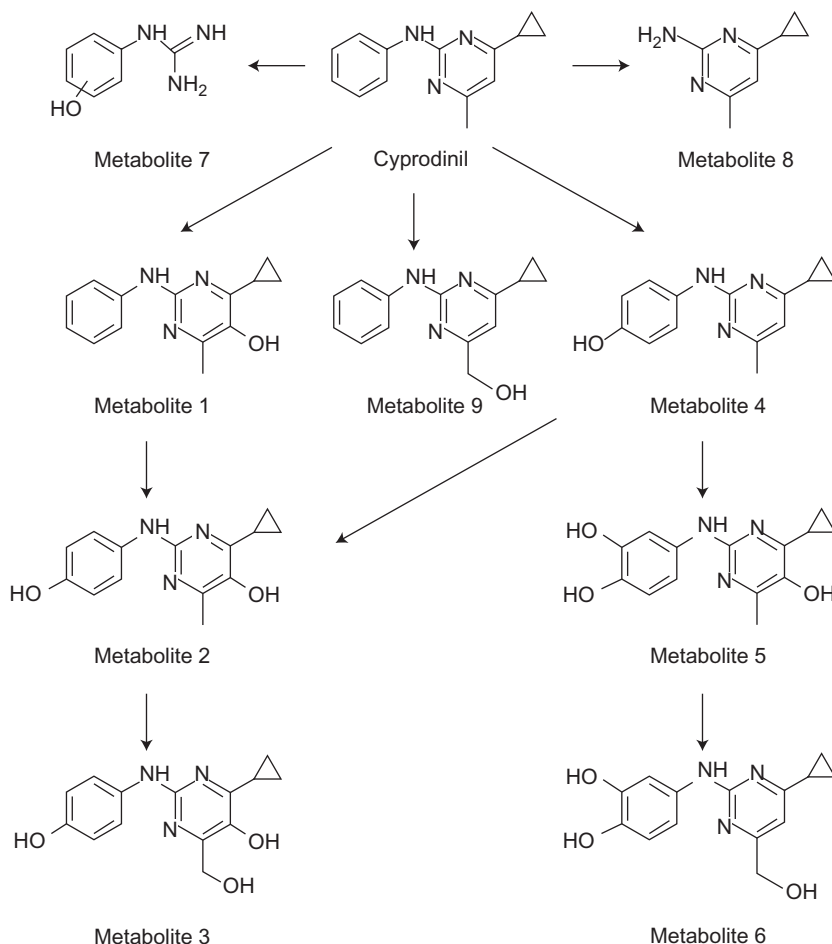


FIGURE 55.1 Phase I metabolism of cyprodinil in rats. Reproduced with permission from Waechter *et al.* (2010).

is metabolized by sequential oxidation of the phenyl and pyrimidine ring (Figure 55.1). In urine and feces, there is no difference in the metabolite patterns of the phenyl or pyrimidyl labeled cyprodinil. Seven urinary, two biliary and two fecal metabolites have been identified, which in total account for 65–80% of the administered dose. Cyprodinil is absorbed in goats to a lesser extent and more slowly than in rats. The major route of excretion is in urine and feces, whereas excretion via milk is minimal. In laying hens, cyprodinil is rapidly and completely eliminated. Residues in eggs and edible tissues are very low. The metabolic pathways of cyprodinil in lactating goats and laying hens are similar to those observed in rats (Figure 55.1) (Waechter *et al.*, 2010).

Carbamic acid derivative fungicides, such as EBDCs, are only partially absorbed, and then they are rapidly metabolized and excreted with no evidence of long-term bioaccumulation. Absorption of oral doses is rapid, and doses are excreted within 24h with approximately half eliminated in the urine and half in the feces. Their common metabolite is ethylenethiourea (ETU). Only

low-level residues are found in tissues, particularly the thyroid. Another compound in this class, propamocarb, is rapidly and nearly completely absorbed and distributed with a concentration reaching peak levels within 1h. Elimination from tissues is rapid; with a half-life ranging from 11 to 26h, urine is the main route of excretion (~75–91% within 24h). Up to 6% of the administered dose is excreted in feces. Propamocarb is extensively metabolized, and only small quantities are unchanged in urine. Metabolism involves aliphatic oxidation of the propyl chain (to form hydroxyl propamocarb) and N-oxidation and N-demethylation of the tertiary amine resulting in propamocarb N-oxide and mono demethyl propamocarb, respectively. Both benomyl and carben-dazim are well absorbed after oral exposure (80–85%) but poorly absorbed after dermal exposure (1 or 2%) in rats, mice, dogs and hamsters. The major pathway of clearance is urinary elimination in rats and mice, but in dogs the majority of the dose (83.4%) is eliminated via feces, with only 16.2% of the dose eliminated in the urine after 72h of dosing. In animals, benomyl is converted

into carbendazim through the loss of the *n*-butylcarbamy side chain prior to further metabolism. In dogs and rats, carbendazim undergoes aryl hydroxylation–oxidation at the 5 and 6 positions of the benzimidazole ring, followed by sulfate or glucuronide conjugation before elimination. The urinary excretion half-life of carbendazim in both male and female rats is approximately 12h. Benomyl or carbendazim or their metabolites are cleared rapidly from blood and exhibit minimal potential for bioaccumulation in rats exposed orally or intravenously (Gardiner *et al.*, 1974; JMPR, 2005).

Similarly, amide fungicides are rapidly absorbed and eliminated. Metalaxyl-M and metalaxyl can lead to stimulation of hepatic and renal cytochrome P450 and some other drug metabolizing enzymes. Tolyfluanid is rapidly and extensively absorbed, followed by rapid metabolism and almost complete excretion, mainly in the urine and to a lesser extent in the bile, within 48h. High tissue concentration has been seen soon after dosing in the kidney and liver, with lower concentrations in the perirenal fat, brain, gonads and thyroid. In most species, the concentration of fluoride in the bone and teeth increases in a dose-related manner (JMPR, 2002, 2005).

After oral dose, conazole fungicides such as triadimenol and triadimefon are rapidly absorbed and widely distributed in liver and kidney. Excretion and metabolism is rapid and extensive, predominantly through oxidation of the *t*-butyl methyl group. Propiconazole indicates rapid and extensive absorption (80% of the administered dose) and is widely distributed, having the highest concentration in liver and kidney. Excretion is more than 95% in the urine and feces within 48h. There is extensive enterohepatic recirculation. The compound is extensively metabolized with oxidation of propyl side chain, hydroxylation of phenyl and triazole rings, and conjugation. The cleavage of dioxolane is significantly different according to species and sex (JMPR, 2004). The other compound, fludioxonil, is rapidly and extensively (80%) absorbed, widely distributed, extensively metabolized and rapidly excreted, primarily in feces (80%), with a small amount being excreted in the urine (20%). The maximum blood concentration is reached within 1h after administration. Elimination is biphasic, with half-lives of between 2 and 5h for the first phase and between 30 and 60h for the second phase. The compound is extensively metabolized, involving primarily oxidation of the pyrrole ring (57% of the administered dose) and a minor oxo-pyrrole metabolite (4% of the administered dose), followed by glucuronyl and sulfate conjugation. There is no potential of accumulation in the tissues. Trifloxystrobin is rapidly absorbed (66%) in 48h and is widely distributed, with highest concentrations in blood, liver and kidney. Within 72h, 72–96% of the administered dose is eliminated in the urine and feces. Metabolism is extensive, and the compound undergoes

hydroxylation, O-demethylation, oxidation, conjugation, chain shortening and cleavage between glyoxyphenyl and trifluoromethyl moieties (JMPR, 2004).

## MECHANISM OF ACTION

There are a series of biochemical changes or free radical-mediated processes; some may also be produced by other mechanisms that have been used to assess tissue injury. This is exemplified by the phenomenon of lipid peroxidation, which has been invoked as a toxic mechanism in many situations and also occurs subsequent to cell death and membrane lysis. However, in most situations, it is difficult to identify the exact mechanism of action. For example, in fungicides containing mercury, the mercury ions inhibit the sulfhydryl group of enzymes involved in the transfer of amino acids across the blood–brain barrier and then interfere with protein synthesis. Organomercurials can also release some mercury ions in the body, but their toxicity is not believed to be a primary action of mercury ions (Sandhu and Brar, 2009). There are several theories regarding the mechanism by which sulfur produces its toxic action. The oxidized sulfur theory attributes toxicity to its oxidation products, such as sulfur dioxide, sulfur trioxide, thiosulfuric acid, or pentathionic acid. The reduced sulfur theory ascribes toxicity to hydrogen sulfide. Direct action theory suggests toxicity due to crosslinking of proteins, formation of other cellular components by free radicals of sulfur or polysulfides, or extensive oxidation of thiol groups leading to loss of function or structural integrity of proteins. Pentachlorophenol (PCP), a halogenated substituted monocyclic aromatic, acts cellularly to uncouple oxidative phosphorylation, with the target enzyme being  $\text{Na}^+/\text{K}^+$ -ATPase. Oxygen consumption is increased, whereas adenosine triphosphate (ATP) formation is decreased. The energy is lost as heat instead of being stored as high-energy phosphate bonds. The electron transport chain responds by using increasingly more available oxygen (increased oxygen demand) in an effort to produce ATP, but much of the free energy is lost as body heat. This leads to depletion of energy reserves (Eaton and Gallagher, 1997). Similarly, organotin compounds, particularly triethyltin, uncouple oxidative phosphorylation, whereas other agents (e.g., sulfur) in the presence of sulfiting agents such as sulfur dioxide uncouple oxidative phosphorylation. Thiamine is cleaved into its constituent pyrimidine and thiazole moieties, rendering it inactive.

Although the biochemical and molecular mechanism(s) by which captan and its analogs exert their cellular toxicity has not been fully established, captan is known



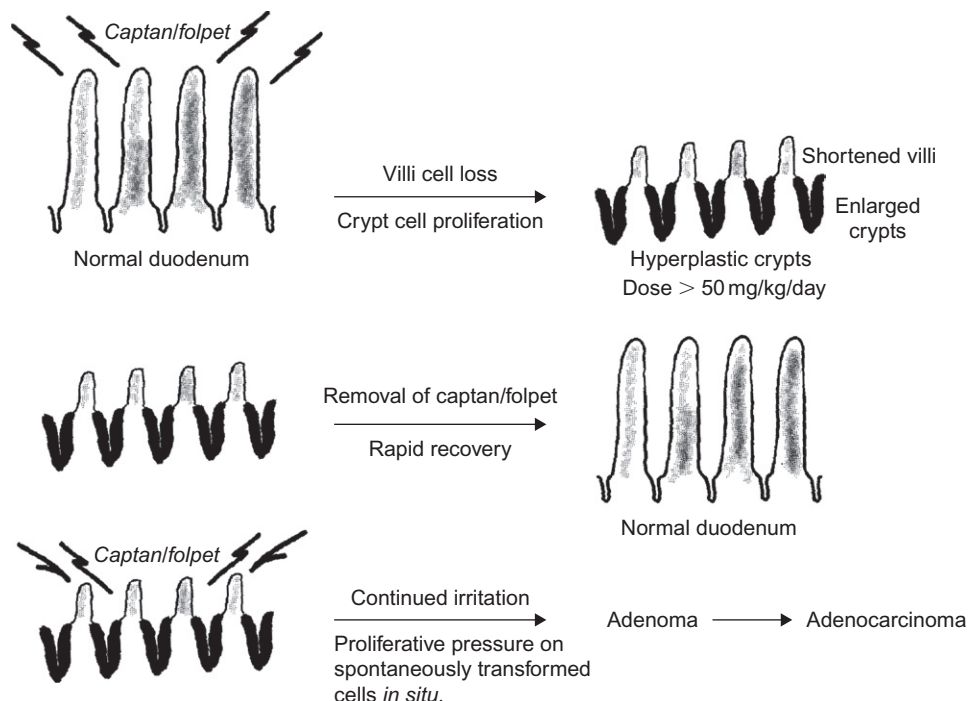


FIGURE 55.2 Mode of action for captan and folpet in the mouse duodenum. Reproduced with permission from Gordon (2010).

to react with cellular thiols to produce thiophosgene, a potent and unstable chemical capable of reacting with sulfhydryl-, amino-, or hydroxyl-containing enzymes (Cremlyn, 1978). Thiols reduce the potency of captan. A volatile product of captan is responsible for mutagenic activity, the intermediate being short-lived and formed more quickly at higher levels at an alkaline pH. There are several other mechanisms by which these chemicals can induce cellular toxicity. For example, mouse tumors develop with oral administration above a threshold if maintained for at least 6 months. As shown in Figure 55.2, epithelial cells that comprise the villi are damaged by exposure to captan and sloughed off into the intestinal lumen at an increased rate. Second, the basal cells in the crypt compartment that normally divide at a rate commensurate with the normal loss of villi cells from the tips of the villi increase, resulting in high cell proliferation, which is not carcinogenic per se but does play a role in tumor development. However, there appears to be no treatment-related duodenal tumor incidence of captan in rats or dogs. Some of the data have been compiled in reviews, and a task force and framework have been evolving for evaluation of the mode of toxicity and tumorigenicity findings in mice bioassay and human relevance for risk assessment purposes (Arce *et al.*, 2010; Cohen *et al.*, 2010; Gordon, 2010).

Chlorothalonil fungicide is a reactive molecule toward thio (–SH) groups. It is a soft electrophile with a

preference for sulfur nucleophiles rather than nitrogen/oxygen nucleophiles. Such chemicals tend to show reactivity toward protein containing critical S electrophiles rather than toward DNA (containing critical O and N nucleophiles). A mechanistic interpretation for the carcinogenicity of chlorothalonil has been published by Wilkinson and Killeen (1996). Repeated administration of chlorothalonil causes hyperplasia in the forestomach of rats and mice. The data are consistent with a temporal sequence of events starting with increased cell proliferation, multifocal ulceration and erosion of the forestomach mucosa, regenerative hyperplasia and hyperkeratosis, and ultimately progressing to the formation of gastric tumors within the forestomach. In dogs, there is no evidence of either neoplastic development or the occurrence of pre-neoplastic lesions in the kidney or stomach. The absence of stomach lesions in dogs is attributable to the anatomical differences between rodents and dogs – dogs do not possess a forestomach. Continued administration of chlorothalonil leads to the development of a regenerative hyperplasia within the renal proximal tubular epithelium. Continued regenerative hyperplasia ultimately results in progression of the kidney lesion to tubular adenoma and carcinoma. Initial cytotoxicity and regenerative hyperplasia within the proximal tubular epithelium are essential prerequisites for subsequent tumor development. The proposed mode of action for the induction of renal toxicity in rodents is outlined in Figure 55.3.



**TABLE 55.1 WHO recommended categories (hazardous nature) of major chemical classes of technical-grade fungicides with representative examples of LD<sub>50</sub> values in rats**

Chemical class	Category	LD <sub>50</sub> (mg/kg BW)
<i>Halogenated substituted monocyclic aromatics</i>		
Chlorothalonil	U	>10,000
Tecnazene	U	>10,000
Dicloran	U	4,000
HCB	Ia	<sup>d</sup> 10,000
Quintozene	U	>10,000
Dinocap	III	980
Dichlorophen	III	1,250
PCP	Ib	<sup>c</sup> 80
Chloroneb	O	–
<i>Chloroalkylthiodicarboximides</i>		
Captan	U	9,000
Captafol	Ia	5,000
Folpet	U	>10,000
<i>Anilinopyrimidines</i>		
Mepanipyrim	U	>5,000
Pyrimethanil	U	4,150
Cyprodinil	III	>2,000
<i>Carbamic acid derivatives</i>		
Ferbam	U	>10,000
Thiram	III	560
Ziram	III	1,400
Propamocarb	U	8,600
Maneb	U	6,750
Mancozeb	U	>8,000
Zineb	U	>5,000
Nabam	II	395
Metiram	U	>10,000
<i>Benzimidazoles</i>		
Benomyl	U	>10,000
Thiophanate-methyl	U	>6,000
Carbendazim	U	>10,000
Fuberidazole	II	336
<i>Conazoles</i>		
Cyproconazole	III	1,020
Diniconazole	III	639
Etridiazole	III	2,000
Hexaconazole	U	2,180
Penconazole	U	2,120
Triadimefon	III	602
Triadimenol	III	900
Azaconazole	II	308
Bromuconazole	II	365
Propiconazole	II	1,520
Tetraconazole (oil)	II	1,031
Imazalil	II	320
<i>Morpholines</i>		
Dodemorph (liquid)	U	4,500
Fenpropimorph (oil)	U	3,515
Tridemorph	II	650
<i>Amides</i>		
Fenhexamid	U	>5,000
Benalaxyl	U	<sup>c</sup> 4,200
Metalaxyl	III	670
Flutolanil	U	>10,000
Tolylfluanid	U	>5,000
Dichlofluanid	U	>5,000
<i>Others</i>		
Thiabendazole	U	3,330
Cycloheximide	O	–
Fludioxonil	U	>5,000
Dimethomorph	U	>5,000
Trifloxystrobin	U	>5,000
Fenpyroximate	II	245

Ia, extremely hazardous; Ib, highly hazardous; II, moderately hazardous; III, slightly hazardous; U, unlikely to present acute hazard in normal use; O, obsolete or discontinued; c, variability; d, dermal. Compiled from IPCS (2002) and JMPR (2005).

had been in use throughout the 19th and early 20th centuries. Elemental sulfur and crude lime sulfur (calcium polysulfide and barium polysulfide) are commonly used as fungicides. The most notable chemical property of sulfur is its tendency to spontaneously oxidize. This property is responsible for sulfur's effects on the eyes, skin and respiratory tract. Safety concerns presented by combustion of sulfur can be mitigated by avoiding its use during periods of high ambient temperature. In general, the use of sulfur does not present a toxicological problem. Only micronized sulfur is responsible for sulfur poisoning. Lethal doses are as follows: cattle, 100–1000 mg/kg body weight (BW); sheep, 1000–1500 mg/kg BW; and horses, 1000–1500 mg/kg BW. The most affected animals are cattle, sheep, goats and dogs. In ruminants, excessive sulfur ingestion may lead to polioencephalomalacia, which is also called cerebrocortical necrosis. It involves softening of gray matter of the brain. Sulfur poisoning may lead to GI tract, neurological and pulmonary effects. On postmortem, congestion of the stomach and intestine, hemorrhagic effusions and petechiae along the GI tract and occasionally on the surface of the bladder have been observed (Jensen *et al.*, 1956; Low *et al.*, 1996; Gammon *et al.*, 2010). Negative effects in humans, especially on the skin, eyes and respiratory tract, have been reported (Gammon *et al.*, 2010). Currently, its use has declined due to the availability of organic fungicides. Another fungicide, barium polysulfide, after reaction with gastric acid yields barium chloride, which is a super purgative (Lorgue *et al.*, 1996; Ballantyne, 2004; Sandhu and Brar, 2009).

## Metallic fungicides

Inorganic metallic fungicides were first used in agriculture. They are protective and preventive. Mercuric and mercurous compounds have been withdrawn because of their toxicity. Ethylmercury phosphate, 2-methoxyethylmercury chloride, phenylmercury chloride and phenylmercury acetate are used as seed treatments for cereals and fodder beet (Lorgue *et al.*, 1996). Cattle, sheep and pigs are the most affected species. Oral LD<sub>50</sub> ranges from 10 to 500 mg/kg BW, depending on the species and the particular compound involved. The organic metallic fungicides are both aliphatic and aromatic. Many are of moderate to high mammalian toxicity, with several being immunotoxic and neurotoxic. Livestock exposed to mercurials may develop central nervous system (CNS) and skin changes. The CNS signs include incoordination and ataxia, body swaying followed by prostration, convulsions and death. CNS stimulation is seen in calves, dogs, cats, rats and humans. However, other species (adult cattle, swine and fowls) manifest CNS depression. Other signs associated with organomercurials are

TABLE 55.2 Select fungicides that cause development toxicity in experimental animals

Chemicals	Malformations
Ammonium salts, manganese and zinc	Multiple defects (rats)
Benomyl	Skeletal malformations, increased mortality (rats), multiple anomalies (mice), small renal papillae but no malformations (rabbits)
Bis(tri- <i>n</i> -butyltin)oxide	Cleft palate and development toxicity (rats, mice)
Bitertanol	Tail, palate, jaw, eye defects (rat)
Captan	CNS, rib, tail and limb defects (hamsters); no teratogenic effects in other four species
Carbendazim	Multiple defects, CNS and rib (rabbits, hamsters)
Conazoles	Limb malformations, postnatal behavior alterations, postural reflex, open field behavior (rat)
Cycloheximide <sup>a</sup>	Variable multiple defects <sup>c</sup>
Dinocap <sup>b</sup>	Skeletal defects, dactyly, hydrocephaly or other development defects
EBDCs (maneb and zineb metabolites, monosulfide and ETU)	Multiple malformations, hyprocephaly (rabbit)/experimental teratogen
Fenpropimorph	Multiple malformations and embryo–fetotoxic effects
Ferbam	Development toxicity and malformations
Fusilazole	Soft tissue and skeletal (rats)
Hexachlorobenzene	Multiple malformations
Mancozeb	Variety of defects, renal and palate (hamsters, mice), rib variation and reduced weight (rats)
Methylmercury	Variety of defects
Polycarbacin	Multiple malformations and embryotoxic
Prolineb	Embryotoxic, malformations
Propiconazole	Multiple malformations
Thiram	Development toxicant
Triadimenol	Multiple malformations
Triadimefon	Developmental toxicity
Tridemorph	Scapula malformations (rabbits)
	Cleft palate, other malformations and development toxicity (rats, mice)

EBDCs, ethylenebisdithiocarbamates; ETU, ethylenethiourea; from Gupta (2011).

<sup>a</sup>Obsolete.

<sup>b</sup>Withdrawn by manufacturer.

<sup>c</sup>Variable: results need further study.

TABLE 55.3 Select fungicides responsible for reproductive problems/malformations in humans

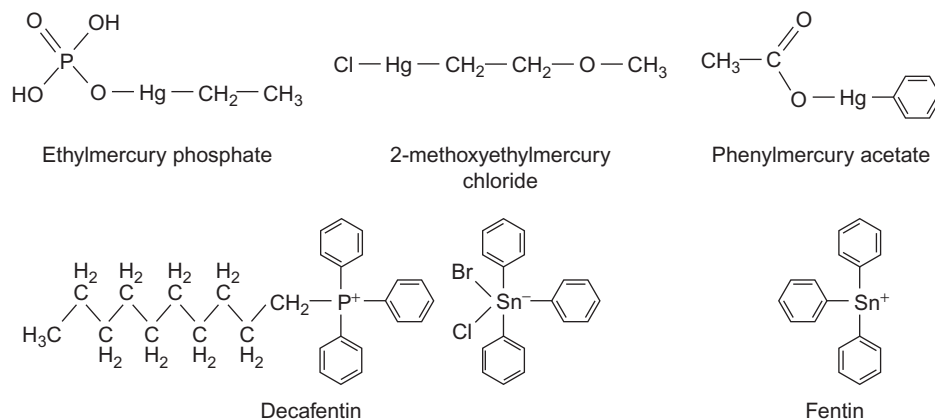
Chemical	Locations	Years	Reproductive abnormalities
Methylmercury	Japan, New Mexico, Iraq, Sweden and Russia	1953 and subsequent years	Disrupts CNS developmental processes in the unborn and infantile
Benomyl	England and Wales	1990s	Tetramelia, eye defects, miscarriages, premature delivery and infants with congenital anomalies
Captan alone and/or with other pesticides	England	1990s	Miscarriages, premature delivery and infants with congenital anomalies
Hexachlorobenzene	Turkey	1955–1957	Stillbirths and neonatal mortality
Zineb		1970s	Reproductive problems

From Gupta (2011).

bronchopneumonia, hyperpyrexia, epistaxis, mucous membrane hemorrhages, hematuria and bloody feces. Skin lesions include eczema, pustules, ulceration, keratinization, dehydration, weakness and death (Sandhu and Brar, 2009). Typical organotin compounds are di- and trialkyl and triphenyl tins. In general, they are severely irritant to the

skin, eyes and mucosae, and several are hepatorenotoxic and immunotoxic. They have been shown to increase susceptibility to infection, decrease lymphopoiesis and decrease T lymphocyte production. Trialkyl tins cross the blood–brain barrier and are centrally neurotoxic (WHO, 1990).





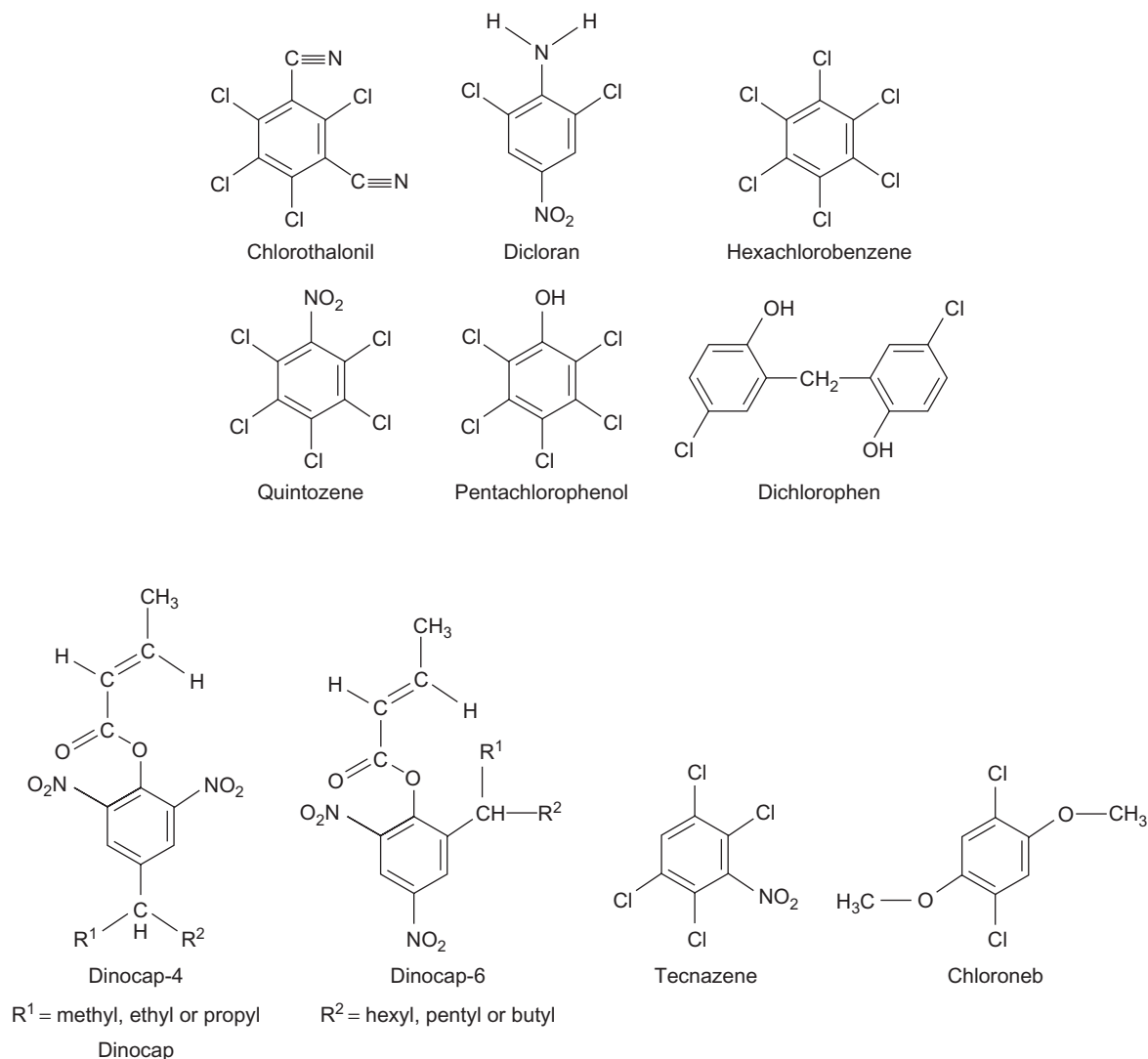
### Halogenated substituted monocyclicaromatics

This class of chemicals includes chlorothalonil, dicloran, HCB, quintozene, PCP, dichlorophen, dinocap, tecnazene and chloroneb. Chlorothalonil is a nontoxic halogenated benzonitrile fungicide (Table 55.1). Dermal irritation has been observed following repeated exposure in the rat and rabbit, indicating the potential for chlorothalonil to cause skin irritation. Chlorothalonil causes irreversible and severe ocular lesions in rabbits. Signs of toxicity include decreased body weight and decreased hematological parameters, increased absolute kidney weight, vacuolar degeneration in the proximal tubular epithelium and hyperplasia of the forestomach. Chlorothalonil is not genotoxic *in vivo*. Treatment-related increase in renal tubular adenoma and carcinoma, squamous cell adenoma and carcinomas of the forestomach of both species have been reported. Chlorothalonil is not a developmental or reproductive toxicant when tested up to doses that cause significant maternal toxicity and maternal death; however, a significant increase in the incidence of postimplantation loss due to early embryonic death has been observed (Parsons, 2010). Tecnazene has an oral rat  $\text{LD}_{50}$  of 2047 mg/kg BW and is a mild irritant in the rabbit eye. The compound has the potential to produce pulmonary adenoma, but it is neither embryotoxic nor teratogenic in mice (Ballantyne, 2004). Chloroneb, quintozene and dicloran have low toxicity (Table 55.1), and dinocap is a moderate eye irritant and has a human skin sensitizing potential. HCB, like other organochlorine compounds, possesses all the properties of chemical stability, slow degradation and biotransformation with potential to accumulate in adipose tissues and organs containing a high content of lipid membranes, and it has the ability to induce a range of tissue cytochrome P450 as well as conjugate enzymes. Repeated exposure in animals results in hepatomegaly and porphyria as well as focal alopecia with itching and eruptions, followed by pigmented scars, anorexia and neurotoxicity expressed as irritability, ataxia and tremors. The compound can

increase hepatic and thyroid tumors in hamsters and is teratogenic in mice (renal and palate malformations) and in rats (increased incidence of 14th rib). HCB is also toxic to developing perinatal animals (Table 55.2) and causes adverse effects on the immune system (Costa, 2008). PCP has oral rat  $\text{LD}_{50}$  of 150–210 mg/kg BW. Common signs of toxicity are increased breathing rate, increased temperature, tremors, convulsions, loss in righting reflex and asphyxial spasms. Corneal injury may result from splashes or vapor overexposure. Toxicity is more often due to contamination of commercial-grade PCP. Technical-grade PCP causes hepatic porphyria, increased microsomal monooxygenase activity and increased liver weight. Lethal dose in cattle and sheep is in the range of 120–140 mg/kg BW. Chronic toxicity leads to emaciation, weight loss, reduced productivity (milk, eggs, meat, etc.) and increased mortality in poultry (ATSDR, 1994; Lorgue *et al.*, 1996).

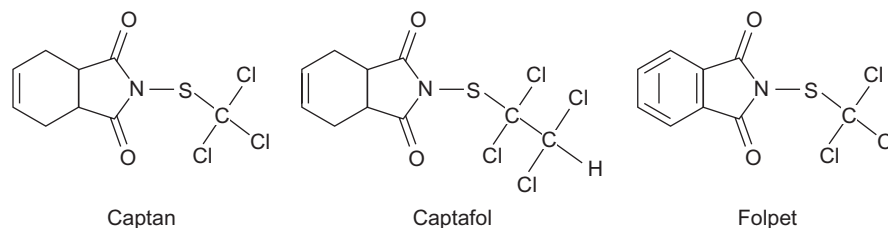
### Chloroalkylthiodicarboximides (phthalimides)

This class of chemicals contains broad-spectrum fungicides (captan, folpet, captafol, etc.) used as surface protectants on many crops. They are usually nontoxic to mammals. The oral  $\text{LD}_{50}$  in rats is as follows: captan, >9000 mg/kg BW; folpet, >10,000 mg/kg BW; and captafol, >5000 mg/kg BW (Table 55.1). However, a low-protein diet makes the animal more sensitive. Of this class of chemicals, folpet and captafol, which are true phthalimides, have been deregistered; only captan, being structurally different (see structural formulae) with a cyclohexene ring, is being used. The compound is a severe eye irritant because of its high reactivity. Folpet induces incidences of diarrhea, vomiting, salivation, reduced food intake and reduced body weight gain. Testes weights are reduced in dogs. A single dose applied to the skin results in mild to low irritation. Long-term exposure to rats causes hyperkeratosis and acanthosis of the esophagus and stomach, particularly



after folpet exposure. Among ruminants, cattle are the most affected, and captan produces toxicity (oral TD, 250–500 mg/kg BW) with labored respiration, anorexia, depression, hydrothorax, ascites and gastroenteritis (Sandhu and Brar, 2009). Mutagenicity may be associated with these agents *in vitro* at exceptionally high doses required to elicit biological effects. These compounds degrade extremely rapidly in the presence of thiols, with a plasma half-life of only a few seconds. However, duodenal tumors in mice have been reported, which were considered to be due to the irritation potential of compounds to the GI tract of rats (Gordon, 2010). Some compounds of this class cause developmental effects (Table 55.2), whereas others are not proven because of, and/or masked by, maternal toxicity and possible nutritional deficits (Costa, 2008). Captan induces hyperplasia of the crypt cells. Following

treatment with folpet, immune function is reduced, villi length is reduced and crypt compartments are expanded, thereby reducing the villi-to-crypt ratio in mice (JMPR, 1990; Tinston, 1995; Waterson, 1995; Gordon, 2010). The most characteristic pathologic finding consists of necrotizing and proliferative changes in the nonglandular portion of the stomach, dilation of the small intestine and focal epithelial hyperplasia in the proximal part of the small intestine in mice following treatment with captan (Gordon, 2010). Captafol differs from captan and folpet in a number of ways, including structure and chemical activity. Both of them have low acute toxicity. They are not carcinogenic, mutagenic, or teratogenic. They are neither selective developmental toxins nor reproductive toxins. They are an irritant of mucus membranes, especially of skin after repeated exposures (Gordon, 2010).

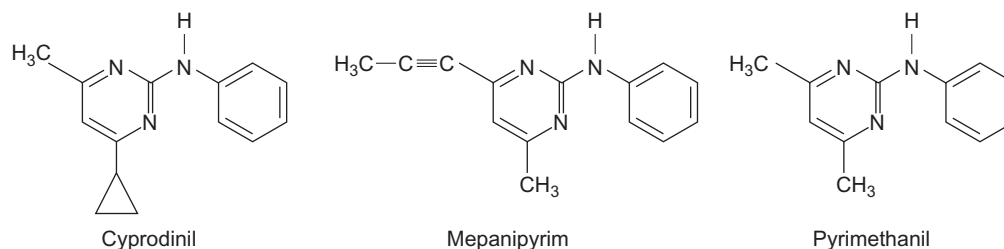


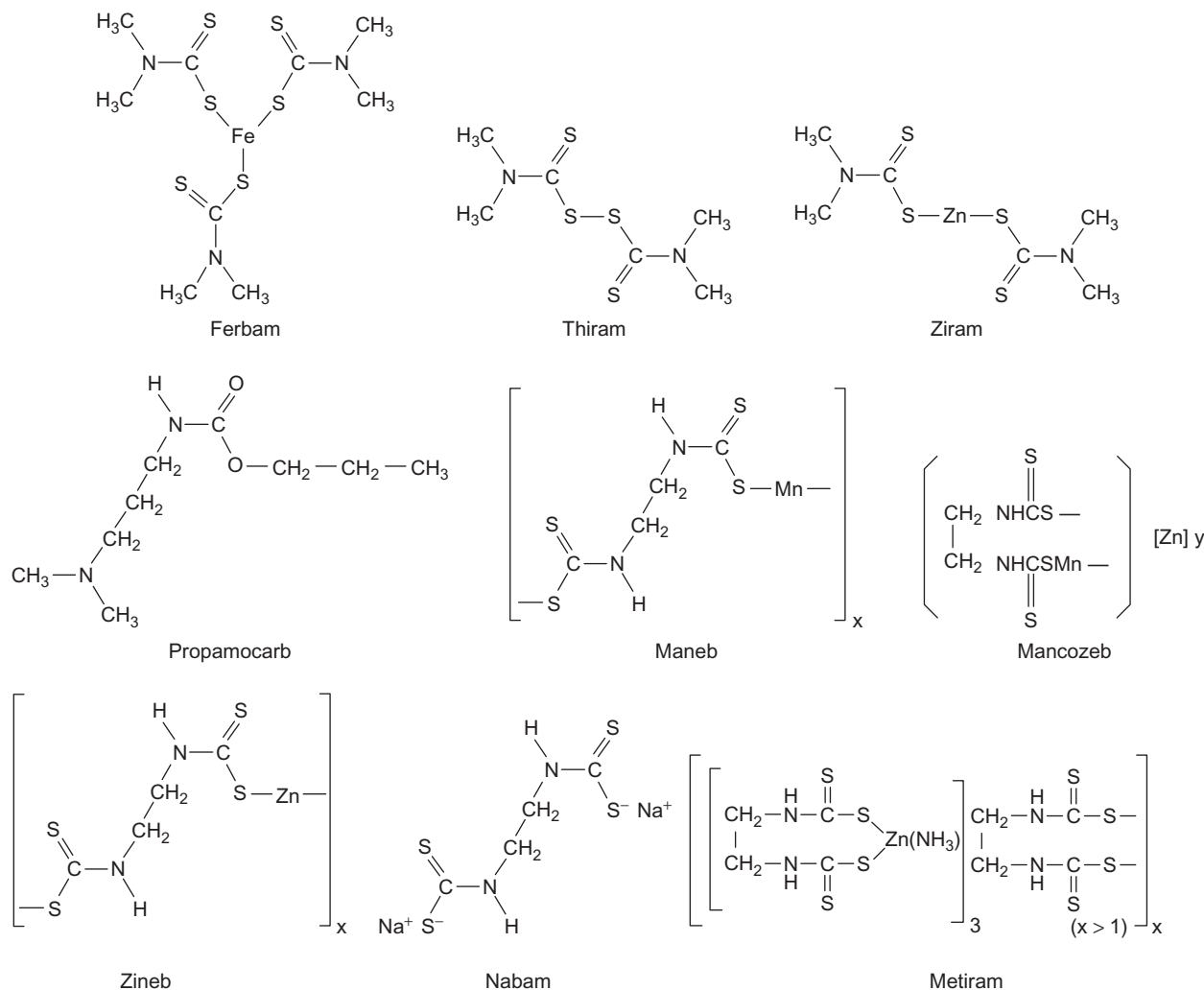
## Anilinopyrimidines

The anilinopyrimidine class of fungicides includes cyprodinil, mepanipyrim and pyrimethanil. The compounds have low toxicity and are unlikely to present acute hazards in normal use (Table 55.1). Cyprodinil produces hepatomegaly with hepatocellular hypertrophy and increased thyroid weights associated with follicular cell hypertrophy and hyprochromasia in rats. Subchronic exposure also resulted in kidney lesions in rats. The compound also causes single cell necrosis in male mice and depletion of glycogen in female mice, whereas in dogs, increased blood platelets have been observed at high doses. Mepanipyrim causes hepatocellular fatty vacuolation and lipofuscin deposition in Kupffer cells and hepatocytes of dogs, whereas such changes are not observed in cyprodinil-treated rats (Terada *et al.*, 1998). Pyrimethanil produces thyroid follicular cell tumors in rats and enhancement of hepatic thyroid hormone metabolism, which may be responsible for thyroid tumorigenesis (Hurlety, 1998). The findings in the thyroid were considered to be secondary to liver changes. Enhancement of hepatic thyroid hormone metabolism and excretion are considered to be the mode of action of thyroid tumorigenesis (Hurlety, 1998; Waechter *et al.*, 2010). Cyprodinil induced microsomal protein and cytochrome P450 contents along with ethoxyresorufin *O*-deethylase, pentoxyresorufin *O*-deethylase and lauric acid 11- and 12-hydroxylase, as well as cytosolic glutathione *S*-transferase activities in rats (Waechter *et al.*, 2010). Generically, cyrodinil and mepanipyrim induce the opposite effects on liver and blood lipid parameters in rats. In general, anilinopyrimidines do not have adverse effects on development toxicity. They are neither genotoxic nor have any carcinogenic potential (Waechter *et al.*, 2010).

## Carbamic acid derivatives

The carbamic acid class of fungicides includes dithiocarbamates (ferbam, thiram, ziram, propamocarb, etc.) and EDBC (maneb, mancozeb, zineb, nabam, metiram, etc.). In general, carbamic acid derivatives, except nabam, have low or moderate acute toxicity by the oral, dermal and respiratory routes (Table 55.1). The main features of toxicity include anorexia, diarrhea and flatulence followed by neurological effects, ataxia, muscular contractions and prostration. With repeated ingestion, there is a possibility of cutaneous effects, alopecia and a risk of antithyroid effects, especially with maneb. Certain compounds inhibit ovulation and egg laying (thiram and ziram). On histopathology, hepatic, renal and pulmonary congestion is common. Occasionally, hepatic degeneration, ascites, enteritis and hydrothorax have been observed (Lorgue *et al.*, 1996). Propamocarb is non-irritating to the eyes or skin. It induces sensitization in a Magnusson-Kligman maximization test. The signs of toxicity include hypokinesia, lethargy, hunched posture, body tremors, clonic convulsions, nasal hemorrhages, piloerection, staggering gait and ataxia. Vacuolar changes in various tissues including choroid plexus in the brain and reduction in organ weights have been observed in the rat and dog. Common development and reproductive abnormalities include reduction in copulation index (female rats) and body weight, retardation in ossification (rat) and increased postimplantation loss (rabbit) (JMPR, 2005). The principal target organ upon repeated exposure to EDBC is the thyroid. These fungicides alter thyroid hormone levels and/or weights. The developmental toxicity includes malformations and embryo-fetotoxic effects at maternally toxic dose levels with EBCs in rats (Table 55.2) (Ollinger *et al.*, 2010).





## Benzimidazoles

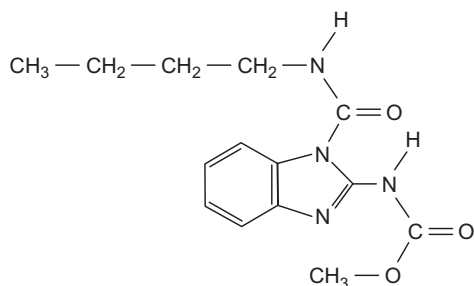
The major benzimidazole fungicides include benomyl, carbendazim and fuberidazole. Benomyl and carbendazim have low toxicity, whereas fuberidazole has moderate toxicity (Table 55.1). Both benomyl and carbendazim produce reproductive and developmental toxic effects in laboratory animals at high oral doses (Table 55.2). Reproductive toxic effects include decreased sperm count, decreased testicular weights and histopathological changes, whereas developmental toxic effects include reduced fetal weight and anomalies of eyes (microphthalmia or anophthalmia), skull and head (hydrocephaly). The effects observed on the oocytes and uterine weight in female rats are direct and are not mediated by endocrine changes (Jeffay *et al.*, 1996; Spencer *et al.*, 1996). A high dose of carbendazim causes an increased incidence of diffuse proliferation of parafollicular cells of the thyroid in female rats. Both of these compounds are not carcinogenic in rats; however,

lifetime exposure in mice shows benign (not malignant), hepatocellular neoplasms and adenomas. Carbendazim is a developmental toxicant and teratogen (JMPR, 2005).

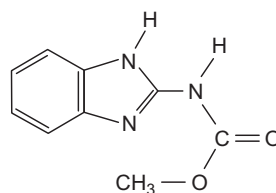
## Conazoles

The conazole class of fungicides includes cyproconazole, diniconazole, triadimefon, triadimenol, propiconazole and imazalil and has low to moderate acute toxicity (Table 55.1). Triadimenol is triazole, and triadimefon is closely related chemically to triadimenol, with increasing toxicity for increasing isomer A ratios (isomer B is less toxic). Triadimenol is non-irritating, whereas technical-grade triadimefon is sensitizing. The other symptoms of toxicity include liver toxicity and CNS effects (general restlessness, alternating phases of increased and reduced motility, and aggressive behavior). Liver adenomas have been observed in female mice. Developmental toxicity indicates increased ovary and testes weights, increased

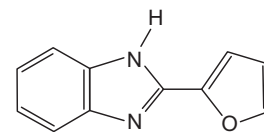




Benomyl



Carbendazim

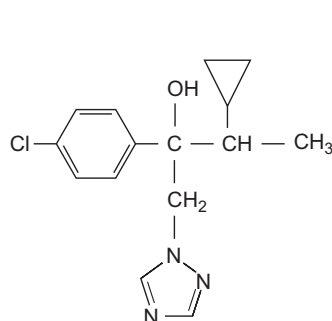


Fuberidazole

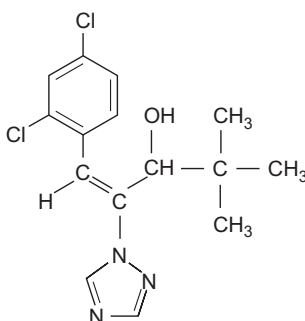
supernumerary lumbar ribs with triadimenol and increased scapula malformations at maternal toxic doses in rabbits after triadimefon (JMPR, 2004). Propiconazole is not an eye irritant in rabbits, but it is irritating to rabbit skin and a skin sensitizer in guinea pigs. The compound causes reduction in body weight, liver toxicity and adverse changes in erythrocytes (rat) and the stomach (dog). On long-term exposure, liver hypertrophy and tumors (mice), uterine lumen dilation (rats), developmental toxicity indicative of reduced pup weight at parentally toxic dose and skeletal variations in laboratory animals have been observed (JMPR, 2004).

## Morpholines

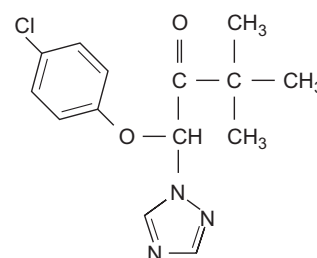
The class of morpholine fungicides includes dodemorph, fenpropimorph and tridemorph. Dodemorph and fenpropimorph are unlikely to cause acute hazards, whereas tridemorph is moderately hazardous (Table 55.1). Dodemorph acetate is moderately irritating to rabbit skin and a severe irritant to rabbit eye. Fenpropimorph is a mild irritant to rabbit skin, whereas tridemorph is non-irritant. Tridemorph and fenpropimorph lead to developmental toxicity (Table 55.2), with an increase in the total number of malformations (JMPR, 2004).



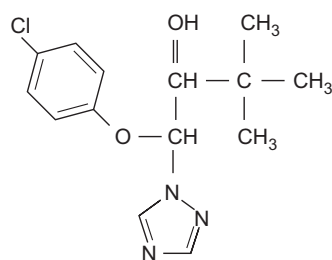
Cyproconazole



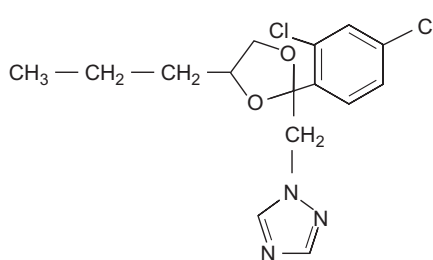
Diniconazole



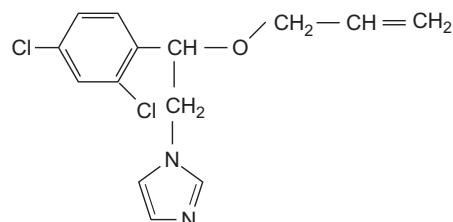
Triadimefon



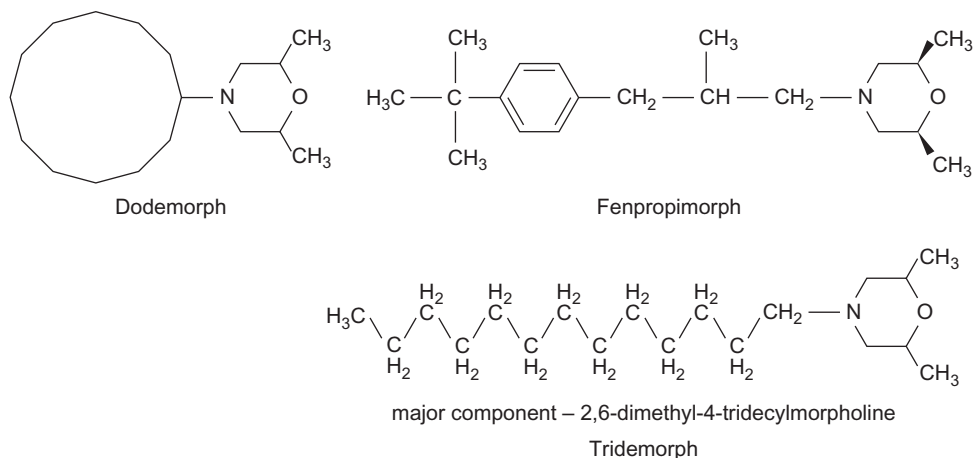
Triadimenol



Propiconazole



Imazalil

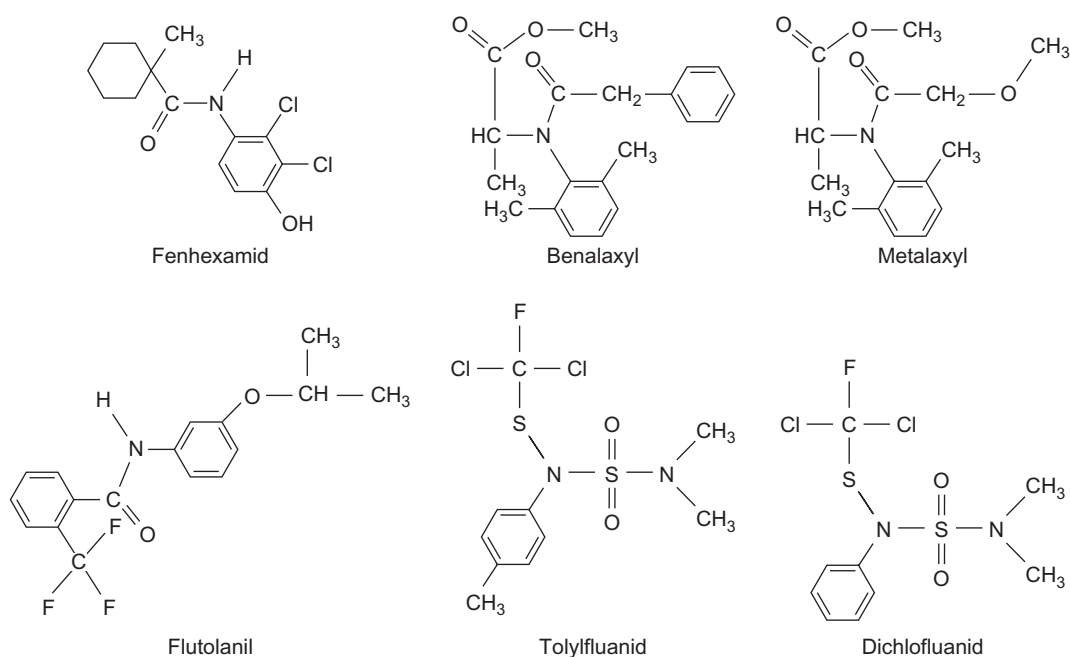


## Amides

Commonly used amide fungicides are fenhexamid, benalaxyl, metalaxyl, flutolanil, tolylfluanid and dichlofluanid. The compounds are of low toxicity, except for metalaxyl, which is slightly hazardous (Table 55.1).

On long-term exposure, benalaxyl causes liver steatosis and hematological changes in rats and atrophy of seminiferous tubules in dogs. In mice, increased mortality associated with amyloidosis has been observed. Reproductive abnormalities in rats include decreased body weight gain, increased liver weight of pups and delayed ossification of cranial bones. Minor skeletal deviations at maternally toxic levels have been reported in rabbits (JMPR, 2005). Metalaxyl is a 1:1 mixture of R-enantiomer and S-enantiomer. Technical-grade metalaxyl-M consists of

a minimum of 97% of the R-enantiomer and 3% of the S-enantiomer. The two compounds are used as fungicides and are severe irritants to rabbits. The dog is the most sensitive species, with the liver as the target organ. Both substances cause hepatocellular enlargement in rats, whereas dogs show changes in blood biochemical parameters indicative of hepatocellular damage (JMPR, 2002). Dichlofluanid and tolylfluanid have a fluorine atom substituted for one of the three chlorine atoms on the trichloromethylthio moiety of captan and folpet. Both compounds do not share a common mechanism of action with captan and folpet with regard to mouse duodenal tumors, principally because they do not induce these tumors. Flutolanil is slightly irritating to the eye. On long-term exposure, it leads to enlargement of the liver, decreased body weight and mild hematological

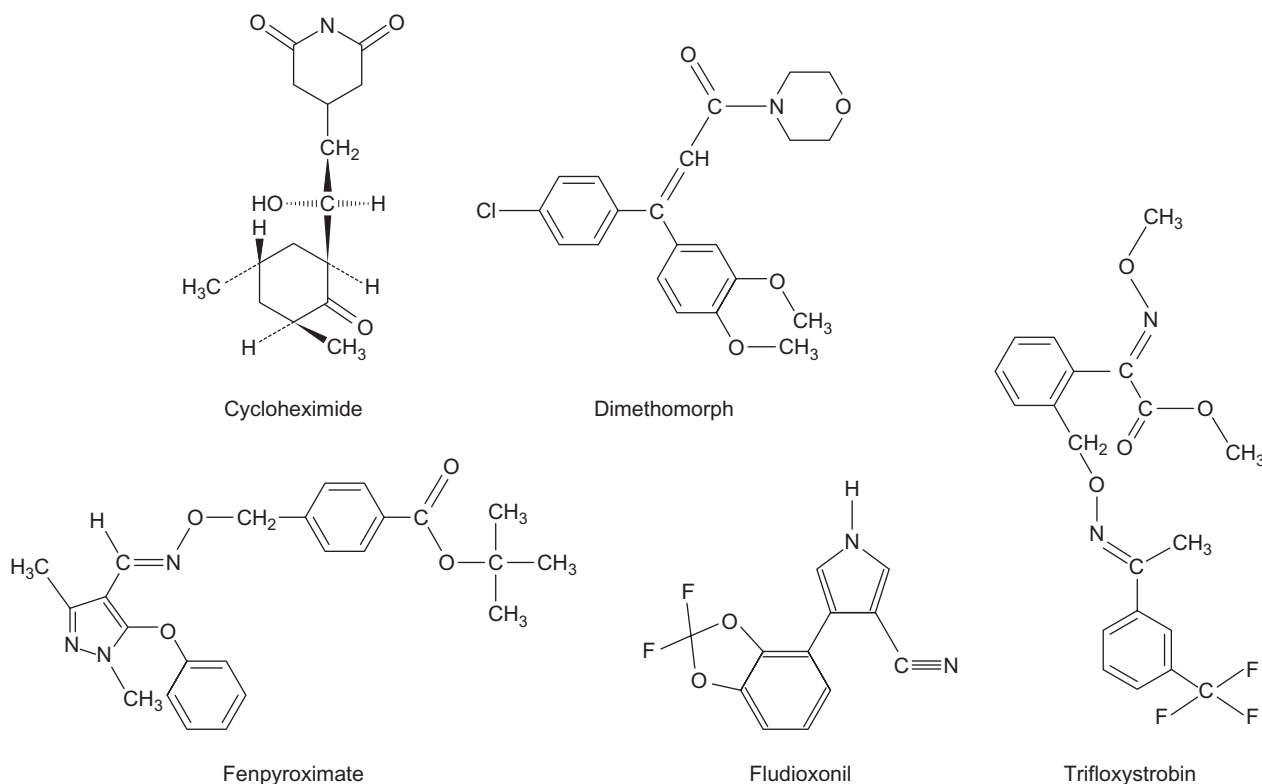


disturbances, with evidence of increased thyroid weight in rats and dogs. On long-term exposure to fenhexamid, the major target organ is the kidney in rats and mice and the hematopoietic system (increase in Heinz bodies) and adrenal gland in dogs. At higher doses, delayed ossification has been observed in rabbits but is not teratogenic (JMPR, 2005). Tolyfluanid is a skin sensitizer and can lead to sedation, decreased motility, disturbed behavior and dyspnea. After intraperitoneal injection, signs consistent with local irritation, altered liver enzyme activity, increased liver weight and histopathological changes that are indicative of liver toxicity in mice, rats and dogs have been reported. At higher doses, signs of renal toxicity and discoloration of bones and teeth, particularly the skull cap and incisors, have been observed. Alterations in thyroid hormone levels have also been observed in a number of studies in rats. The compound causes decreased pup viability at maternally toxic doses but is not teratogenic. These amides are neither genotoxic nor have any carcinogenic potential in animals (JMPR, 2002).

## Others

Several other antibiotic substances, thiocarbonates and cinnamic acid derivatives are used as fungicides. For

example, sodium tetrathiocarbonate, a thiocarbonate fungicide, is moderately toxic. The rat oral LD<sub>50</sub> is 631 mg/kg BW. It is a severe irritant to rabbit skin and a marked irritant to rabbit eyes. Another compound, cycloheximide, is extremely toxic, including development toxicity (Table 55.2), and has mutagenic potential. Dimethomorph has low oral toxicity in rats, is not an irritant to rabbit skin and is a minimal irritant to rabbit eyes. The acute oral LD<sub>50</sub> of fenpyroximate is 245 and 480 mg/kg BW in male and female rats, respectively. The compound may cause developmental toxicity in rats. Fludioxonil, a phenylpyrrole fungicide, and trifloxystrobin have low acute toxicity in rats. Fludioxonil is a slight eye irritant in rabbits, but it is neither a skin irritant in rabbits nor a skin sensitizer in guinea pigs. On long-term exposure, it causes liver necrosis, kidney nephropathy and mild anemia, and blue coloration of the urine and perineal fur. Reproductive toxicity indicates decreased pup weight gains in rats at parentally toxic doses. Trifloxystrobin is non-irritating but may be a skin sensitizer. Toxic symptoms are associated with liver toxicity, changes in kidney weight, atrophy of the pancreas and spleen abnormalities. Development toxicity indicates decreased body weight gain of pups accompanied by delayed eye opening at parentally toxic doses (JMPR, 2004).



## ENDOCRINE DISRUPTION

In both males and females, some fungicides affect reproduction through different mechanisms of action by endocrine disruption: exogenous agents interfere with normal reproduction and development processes. In males, normal reproductive function involves interaction of the hypothalamic–pituitary–testis axis and the thyroid gland. In females, increased concentrations of xenoestrogens may affect ovarian function through the disruption of feedback mechanisms in the hypothalamus–pituitary–gonadal axis (Flaws and Hirshfield, 1997; Bretveld *et al.*, 2006). Fungicides, like other chemicals, may disrupt all stages of hormonal function of the reproductive system. In females, during pregnancy, and to a greater extent during lactation, a portion of the maternal body burden of these chemicals is transferred to the offspring. Fungicides that are toxic to endocrine cells and that delete germ cells are beyond the scope of this chapter. However, it is well known that some fungicides affect male and female reproduction through different mechanisms of action by endocrine disruption. For example, fenarimol, prochloraz and chlorothalonil cause infertility in male and female rats through inhibition of CYP450 enzymes involved in steroid metabolism and alter sexual differentiation through antagonism of the androgen receptor(s) (Ankley *et al.*, 2005; Noriega *et al.*, 2005). The fungicide vinclozalin was shown to have endocrine-modulating effects in male offspring when rat dams were treated during the last one-third of gestation through postnatal day 3. The anogenital distance was reduced, and there were cleft phallus, hypospadias and other malformations in the offspring (Gray *et al.*, 1994). Procymidone has anti-androgenic properties. Long-term exposure of rats to procymidone had different effects on the pituitary–gonadal axis *in vivo* and on Leydig cell steroidogenesis *ex vivo*. The disruption of hormonal feedback could be due to its anti-androgenic action through activation of the endocrine axis, thereby causing hypergonadotropic activation of testicular steroidogenesis (Svechnikov *et al.*, 2005). From experimental studies, it has been concluded that fungicides can disturb the reproduction and development processes of both males and females through endocrine signals in organisms indirectly exposed during prenatal or early postnatal life. Such effects during fetal development may be permanent and irreversible. They may act through hormone receptors and enhance or diminish the activity normally controlled by endogenous hormones. According to one estimate, in 2003, eight fungicides (benomyl, hexachlorobenzene, mancozeb, maneb, metiram complex, tributyltin, zineb and ziram) were identified as potentially causing endocrine disruption in animals. Most of these were identified accidentally rather than as a result of an exhaustive screening process (Pocar *et al.*, 2003).

## TREATMENT

In some cases, there is no treatment, whereas in others supportive therapy as required by condition is indicated: (1) in the initial stages, use of emetics, gastric lavage, or activated charcoal may be helpful for removal of residual material from the GI tract; (2) oxygen should be provided immediately to meet oxygen demand; (3) body temperature should be lowered (use of cool baths, cool sponging, or placing the animal in the shade or in a cool, quiet room); (4) phenothiazine tranquilizers by the intramuscular route are advised to decrease exertion and stress and to facilitate handling of the animal; however, these should not be used to sedate or comatose the animal; (5) balanced electrolyte solution should be administered to prevent dehydration; and (6) the animal should be removed immediately from the exposure site. In the case of metallic poisoning, there is no satisfactory treatment of organomercurial toxicosis in farm or pet animals. Once developed, brain lesions are irreversible, and treatment in such cases is meaningless. Traditional chelators such as dimercaprol (BAL) or sodium thiosulfate have been recommended for many years but have little or no value due to their poor affinity for organomercury compounds (Gupta, 2010b).

## CONCLUSIONS AND FUTURE DIRECTIONS

Fungicides vary widely in chemical structure and thus in toxicity to livestock. The main hazard to livestock from fungicides is likely to arise from their use as dressings for the protection of stored grains, potatoes, etc. A number of livestock poisoning cases result from feeding of treated grains. Most available toxicity data are from laboratory animals; little information is available for farm animals and pets. In general, fungicides have low to moderate toxicity. However, several fungicides, such as alkylthiocarbamic acid (manganese, zinc and ammonium salts), halogenated substituted monocyclic aromatics (dinocap), carbamic acid derivatives (maneb and zineb metabolites and ethylenethiuram monosulfide), ferbam, mancozeb and maneb metabolites, HCB, benzimidazoles (benomyl and carbendazim), bis(tri-*n*-butyltin)oxide, chloroalkylthiocarboximides (captafol and folpet) and tridemorph are known to cause developmental toxicity and oncogenesis. More than 80% of all oncogenic risk from the use of pesticides derives from a few fungicides; only a small number of pesticide-related deaths from fungicides have been reported. Some fungicides are known to disrupt the endocrine system and may lead to reproductive and developmental



abnormalities. Based on teratogenicity, several fungicides including cycloheximide have been deregistered or banned in many countries but are still used in other, less regulated areas of the world. Other fungicides are undergoing re-evaluation because of changing regulatory policies; suspected toxicity, particularly as carcinogenic, mutagenic, or toxic to reproduction; and incomplete or outdated toxicity database.

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## Anticoagulant rodenticides

Michael J. Murphy

### INTRODUCTION

The origin of oral anticoagulant therapy and anticoagulant rodenticides traces back to investigations of moldy sweet clover poisoning in the 1920s. This disease of cattle in Wisconsin was characterized by high mortality and internal bleeding. Investigations revealed that the diseased cattle had been fed moldy sweet clover hay.

An association between vitamin K and coagulopathies was made in the mid-1930s (Dam, 1935; Fieser *et al.*, 1939). Soon thereafter, Professor Link reported the discovery of dicoumarol in the moldy hay (Last, 2002). Naturally occurring coumarin in sweet clover hay is converted by fungi to dicoumarol. Dicoumarol was found to be the causative agent of the disease, so the elements needed for the disease were coumarin-containing plant material plus mold growth. Subsequently, a range of molecules were synthesized. Warfarin became the most popular (Duxbury and Poller, 2001). Warfarin takes its name, in part, from the Wisconsin Alumni Research Foundation. Warfarin and dicoumarol found application as both oral anticoagulants and rodenticides. Sweet clover requires the action of molds to form dicoumarol, whereas giant fennel does not.

Giant fennel, *Ferula communis*, grows in Mediterranean countries. It has a naturally occurring anticoagulant effect. An association between the plant and anticoagulation was first reported in the 1950s (Costa, 1950a,b; Carta, 1951). It was further investigated in Italy (Mazzetti and Cappelletti, 1957; Corticelli and Deiana, 1957; Corticelli *et al.*, 1957; Cannava, 1958) and then Israel (Shlosberg and Egyed, 1983). The anticoagulant activity of the plant in Morocco has been reviewed (Lamnaouer, 1999).

Five coumarins and 11 daucane derivatives have been isolated from *F. communis* (Arnoldi *et al.*, 2004). Previously identified chemicals include allohedycaryol (Zhabinskii, 1996), fercoperol (Miske *et al.*, 1986), and ferulenol.

The toxicity of ferulenol in rats, mice, and sheep has been reported. Ferulenol is a 4-hydroxycoumarin with the expected anticoagulation effects (Fraigui *et al.*, 2002). The single oral LD<sub>50</sub> of ferulenol in albino mice is 2100 mg/kg body weight (BW) (Fraigui *et al.*, 2002). This value is quite similar to oral LD<sub>50</sub> values of 1650 and 2000 mg/kg for rats and mice, respectively, obtained using fessoukh, the resinous gum of *Ferula* (Fraigui *et al.*, 2001). This supported results of prior studies in rats (Aragno *et al.*, 1988; Tagliapietra *et al.*, 1989).

Anticoagulation activity of the plant in sheep was reported in 1985 (Shlosberg and Egyed, 1985). Subsequently, ferulenol has been measured in the serum of sheep experimentally dosed with 600 g of powdered plant material (Tligui *et al.*, 1994). Ferulenol was detected 6 h after dosing and for approximately 12 h after cessation of dosing. The prothrombin time (PT) was elevated to six times normal approximately 70 h after the last dose and returned to normal by day 5 (Tligui and Ruth, 1994; Tligui *et al.*, 1994).

*Ferula* has also been examined for chemotherapy (Poli *et al.*, 2005), antimycobacterial (Appendino *et al.*, 2004; Mossa *et al.*, 2004), and microtubule effects (Bocca *et al.*, 2002) and for testicular and epididymal changes in rams (Gil *et al.*, 2002), much like warfarin and other oral anticoagulants have been examined. It is found in Morocco (Fraigui *et al.*, 2001), Israel (Shlosberg and Egyed, 1983), and Italy (Tagliapietra *et al.*, 1989).

The term oral anticoagulants normally refers to these chemicals when used therapeutically. The oral anticoagulants are briefly discussed before a detailed discussion of the application of the dicoumarol progeny as anticoagulant rodenticides is presented.

Warfarin and its congeners are still used as therapeutic agents. Oral anticoagulants available therapeutically in Europe include warfarin, phenprocoumon, and nicoumalone, which is also called acenocoumarol (Shetty *et al.*, 1993). Oral anticoagulants are used therapeutically to reduce thromboembolic events. Warfarin examples include a reduction in catheter-related thrombosis (Guidry *et al.*, 1991; Magagnoli *et al.*, 2006); early venous thrombosis after operations (Calnan and Allenby, 1975; Pan *et al.*, 2005), including hip surgery; atrial fibrillation (Middlekauff *et al.*, 1995; Reiffel, 2000); and myocardial infarction (Asperger and Jursic, 1970). Dicoumarol is also used in the therapy of thrombotic occlusion of intracoronary stents (Alonso Martin *et al.*, 1997), prosthetic valves (Dalla, 1994), and other types of venous thromboses (Piovela *et al.*, 1995; Ferlito, 1996) or thrombophlebitis (Byrne, 1970; Creutzig, 1993).

Warfarin may also reduce the recurrence of malignant melanoma (Thornes *et al.*, 1994) or small cell lung cancer (Aisner *et al.*, 1992). This anti-metastatic activity may be associated with the distribution of metastatic cells on thrombi (McNiel and Morgan, 1984; Smith *et al.*, 1988).

Low-dose warfarin therapy has been adopted in the United States, Europe, and by the World Health Organization (Duxbury and Poller, 2001). The international normalized ratio (INR) is used to measure anticoagulation (Duxbury and Poller, 2001).

Oral anticoagulants have been used for decades, and a number of adverse events have been recognized. Most are related to drug interactions (Dayton and Perel, 1971). Although controversy exists regarding whether acetaminophen induces an adverse effect on warfarinized patients in therapeutic doses (Toes *et al.*, 2005), the acetaminophen metabolite NAPQI (*N*-acetyl-*para*-benzoquinoneimine) seems to interfere with vitamin K-dependent  $\gamma$ -carboxylase. Such interference may lead to potentiation of the anticoagulant effect of warfarin (Thijssen *et al.*, 2004). COX-2 inhibitors may interfere with control of warfarin anticoagulation in elderly patients (Savage, 2005), and rifampin may interfere with warfarin metabolism due to induction of P450 activity (Strayhorn *et al.*, 1997; Finch *et al.*, 2002). Inconsistent anticoagulation has been recognized in generic versus prescription warfarin (Burns, 1999), and a single case of hemothorax with eosinophilia has been reported in a warfarin overdose (Nasilowski and Krenke, 2002).

Complementary or alternative medical therapies may also interfere with warfarin metabolism (Tumova, 2000; Wood *et al.*, 2003). For example, passionflower,

hydroalcoholic extracts, juniper, and verbena contain vitamin K<sub>1</sub>, so they can lessen the anticoagulation effect of warfarin therapy (Argento *et al.*, 2000). On the other hand, genoderma, japonicum, papaw, *Salvia miltiorrhiza*, ginseng, devil's claw, garlic, quinine, ginkgo, ginger, red clover, and horse chestnut may reinforce the anticoagulant effect of warfarin by various mechanisms (Argento *et al.*, 2000).

A "coumarin-induced hepatitis" has been reported in warfarinized patients (Rehnqvist, 1978; Bint and Burt, 1980; Tanaka *et al.*, 1985; Hohler *et al.*, 1994; Matsukawa *et al.*, 1994; Ehrenforth *et al.*, 1999; Bamanikar and Hiremath, 2002; Biagini *et al.*, 2006). Liver damage has also been reported in a brushtail possum (*Trichosurus vulpecula*) dosed with the anticoagulant rodenticide pindone (Jolly *et al.*, 1994). Pindone has been associated with an increased frequency of sex chromosome loss in *Drosophila melanogaster* (Santoro *et al.*, 1993). The remainder of the chapter is devoted to the application of the chemical progeny of dicoumarol to pest control.

## BACKGROUND

In the 1940s, a small British pharmaceutical company suggested that dicoumarol might have rodenticidal properties. Trials carried out by Armour and Barnett (1950) confirmed the idea and started the era of anticoagulant rodenticides. Warfarin was the first anticoagulant rodenticide introduced into the market soon after World War II and became widely used in many countries. Other anticoagulant compounds with potency similar to that of warfarin were also synthesized. These early anticoagulant rodenticides are often called first-generation anticoagulant rodenticides. These first-generation compounds generally have moderate toxicity, with acute LD<sub>50</sub> values ranging from 10 to 50 mg/kg BW. The first-generation compounds often needed continuous bait exposure for rodent control.

Many rodent species developed a resistance to warfarin presumably due to continued exposure and widespread use (Jackson *et al.*, 1975). Consequently, new chemical structures were synthesized and used as anticoagulant rodenticides. These newer compounds are generally more toxic than warfarin, with acute LD<sub>50</sub>s of 0.2–3.9 mg/kg BW. For example, a bait concentration of only 50 ppm of brodifacoum is adequate to give control in a single feeding for most rodents and non-commensal species (Matolesy *et al.*, 1988). These newer compounds are called second-generation anticoagulant rodenticides, and they are often referred to as "super-warfarins" in the contemporary medical literature



TABLE 56.1 Oral LD<sub>50</sub> values (mg/kg body weight) of some anticoagulant rodenticides

Animal	LD <sub>50</sub> (mg/kg body weight)		
	Bromadiolone	Brodifacoum	Difenacoum
Rat (acute)	0.65	0.27	1.8
Rat (chronic)	(0.06–0.14) × 5	(0.05–0.08) × 5	0.15 × 5
Mouse	0.99	0.4	0.8
Rabbit	1.0	0.2	2.0
Pig	3.0	10.0	80.0
Dog	10.0	3.5	50.0
Cat	25.0	25.0	100.0
Chicken	5.0	10.0–20.0	50.0
Guinea pig	2.8	–	–
Opossum	–	0.17	–
Sheep	–	10.0	100.0

(Chong *et al.*, 1986; Greeff *et al.*, 1987; Swigar *et al.*, 1990; Wallace *et al.*, 1990; Routh *et al.*, 1991; Wilton, 1991; Exner *et al.*, 1992; Rauch *et al.*, 1994; Hui *et al.*, 1996; Tecimer and Yam, 1997; Chua and Friedenber, 1998; Gallo, 1998; Pavlu *et al.*, 2005; Sharma and Bentley, 2005; Dolin *et al.*, 2006).

Anticoagulant rodenticides are also categorized based on chemical structures. The chemical structures of the currently marketed products are classified into two chemical classes: hydroxycoumarins and indanediones.

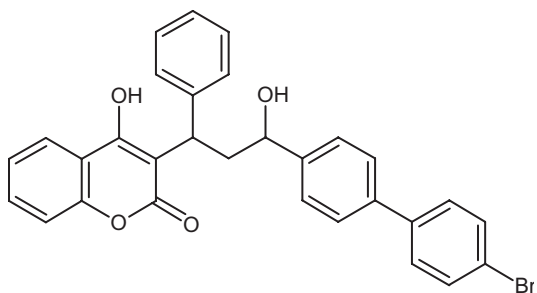
Hydroxycoumarins have a 4-hydroxycoumarin ring with different side chain substituents at the 3-position. Commonly used anticoagulant rodenticides in this group are bromadiolone, brodifacoum, coumatetralyl, coumatetralyl, difenacoum, and warfarin.

Indanediones have a 1,3-indanedione structure with different side chain substituents at the 2-position. The most common anticoagulant rodenticides in this group are chlorophacinone and diphacinone. Each of these compounds are briefly summarized later.

Several representative chemicals are described next. A number of other chemicals have also been developed.

## Hydroxycoumarins

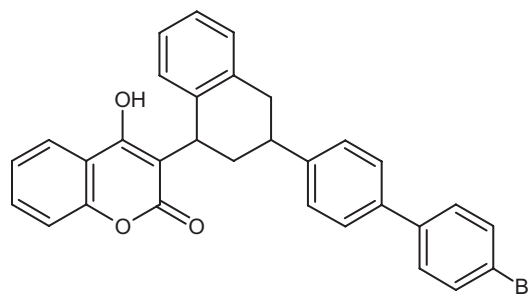
### Bromadiolone



Bromadiolone (3-(3-(4'-bromobiphenyl-4-yl)-3-hydroxy-1-phenyl propyl)-4-hydroxycoumarin) was synthesized and

marketed by the French company Liphia S.A. during the mid-1970s. It is used widely for control of commensal and field rodents in many countries. Technical-grade bromadiolone is 97% pure. It is a yellowish powder and stable up to 2000°C (Chalermchaikit *et al.*, 1993). It is very soluble in dimethylformamide (730 g/L), less soluble in ethyl acetate (25 g/L) and ethanol (8.2 g/L), and sparingly soluble in water (0.019 g/L). Bromadiolone is considered more palatable to rodents than most other anticoagulants. Its concentration in baits is usually 50 ppm (Chalermchaikit *et al.*, 1993). Although bromadiolone is considered a second-generation anticoagulant rodenticide, some resistance problems have been reported with *Rattus norvegicus* and *Mus musculus* in the United Kingdom and Denmark (Lund, 1971; Rowe *et al.*, 1981).

### Brodifacoum



Brodifacoum (3-(3-(4'-bromobiphenyl-4-yl)-1,2,3,4-tetrahydronaphth-1-yl)-4-hydroxycoumarin) is one of the newer and more potent second-generation anticoagulant rodenticides. It was introduced in 1977 by Sorex Ltd. of London and then developed by the Imperial Chemicals Incorporated (ICI) Plant Protection Division (Chalermchaikit *et al.*, 1993).

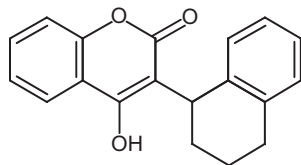
Pure brodifacoum is an off-white to fawn-colored powder with a solubility of 6–20 g/L in acetone, 3 g/L in chloroform, 0.6–6 g/L in benzene, and less than 10 mg/L in water. It is very stable in the environment, with no loss after 30 days of exposure to direct sunlight (Chalermchaikit *et al.*, 1993).

Brodifacoum has been marketed in several countries for the control of a wide range of rodent pest species. It is available as a 0.005% pellet for rat and house mice controls, a smaller 0.001% pellet for field rodent control, and as 29-g wax blocks for sewer rat control. It is the only anticoagulant rodenticide to produce 100% mortality in most rodent species after only a 24-h dose (Chalermchaikit *et al.*, 1993). Brodifacoum was effective against warfarin-resistant rats and mice in 1984, but the possibility of resistance has been raised (Lund, 1984).

There is variation in the susceptibility of species to brodifacoum. The average pig, sheep, or chicken must consume considerable quantities of prepared bait to be endangered. However, dogs are susceptible and are

commonly exposed to potentially toxic quantities of brodifacoum (Chalermchaikit *et al.*, 1993).

#### Coumatetralyl

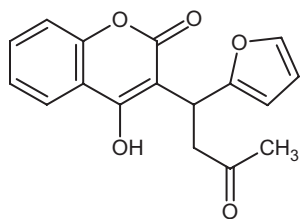


Coumatetralyl (3-(alpha-tetralyl)-4-hydroxycoumarin) was introduced by Bayer AG with the trademark name of Racumin. It has been used for commensal control in many countries. It is formulated as a dry bait (0.0375%), a liquid bait of its sodium salt, and a 0.75% tracking dust (Chalermchaikit *et al.*, 1993).

Pure coumatetralyl is a colorless powder that is stable at temperatures below 150°C. Its solubility is 20–50 g/L in propan-2-ol, 50–100 g/L in methylene dichloride, and 4 mg/L in water. The acute and chronic LD<sub>50</sub>s to *R. norvegicus* are 16.5 and 0.3 mg/kg for five consecutive doses, respectively. Chickens are quite resistant to coumatetralyl, with a chronic LD<sub>50</sub> of 50 mg/kg for eight consecutive doses. Signs did not appear in fish until the concentration of coumatetralyl reached 1000 mg/L in water (Chalermchaikit *et al.*, 1993).

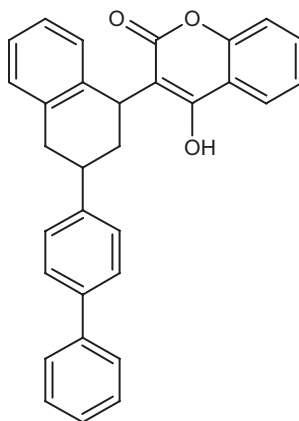
Despite its low toxicity, it is reported to be slightly more effective than warfarin against *R. norvegicus*, apparently due to higher palatability. Coumatetralyl was introduced after the detection of warfarin-resistant rat populations and showed considerable success for a number of years, but resistant pests have been reported in the United Kingdom and Denmark (Rowe and Redfern, 1968; Lund, 1988).

#### Coumafuryl



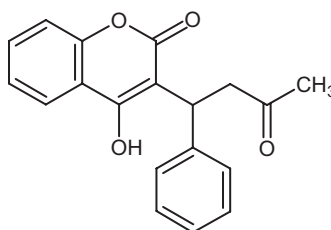
Coumafuryl (3-(alpha-acetyl-2-furyl)-4-hydroxycoumarin) is a German anticoagulant, introduced in 1952, and is used at 0.025–0.05% in baits. Its toxicity is considered equal to that of warfarin for *R. norvegicus* but slightly less efficient against *M. musculus*. The chronic LD<sub>50</sub> in *R. norvegicus* is 1.4 mg/kg for five repeated doses. Cats and dogs seem to be almost as susceptible as rats, with dogs being killed by 2 mg/kg for five repeated doses and cats by 10 mg/kg for four repeated doses (Chalermchaikit *et al.*, 1993).

#### Difenacoum



Difenacoum (3-(3-(p-diphenyl)-1,2,3,4-hydronaphth-1-yl)-4-hydroxycoumarin) was synthesized in the United Kingdom and marketed in 1975 by Sorex Ltd. under the trademark Neosorexa and by ICI Plant Protection Division under the trademark Ratak as a 0.005% pelleted bait and as a wax block. Pure difenacoum is an off-white powder with a solubility of greater than 50 g/L in acetone, 600 mg/L in benzene, and less than 10 mg/L in water. It is more toxic than warfarin, but it is less palatable. Difenacoum is still effective against many populations of warfarin-resistant rats (Desideri *et al.*, 1979), but resistance may be developing in the United Kingdom (Greaves *et al.*, 1982).

#### Warfarin

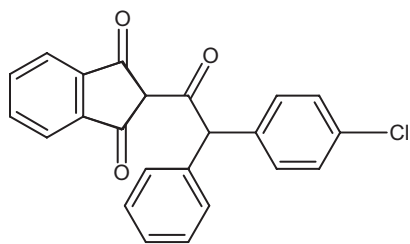


Warfarin (3-(alpha-acetylbenzyl)-4-hydroxycoumarin) was the first anticoagulant rodenticide, introduced soon after World War II after development by the Wisconsin Alumni Research Foundation. Warfarin is still used widely, especially for the control of *R. norvegicus* in areas where resistance has not developed. In its racemic form, warfarin is colorless and crystalline, insoluble in water, but readily soluble in acetone, dioxane, and moderately soluble in alcohols. Warfarin is formulated as dry bait (0.005–0.05%) as well as a liquid bait, based on the sodium salt, and a tracking dust (0.5–1.0%). It is generally applied as the S-isomer, which has a toxicity 10 times greater than that of the R-isomer. The acute and chronic LD<sub>50</sub>s for *R. norvegicus* are approximately 10–12 mg/kg and 0.75 mg/kg for five repeated doses, respectively (Colvin and Wang, 1974).

Warfarin is sometimes combined with an antibacterial agent, sulfaquinoxaline, to reduce the bacterial production of vitamin K in the rat intestine, but the effectiveness of this combination has not been proven. Warfarin is considered one of the safest anticoagulants with regard to domestic and other non-target animals. Serious resistance problems have been reported in Europe. It has been evaluated against sewer rats in London (Channon *et al.*, 2000).

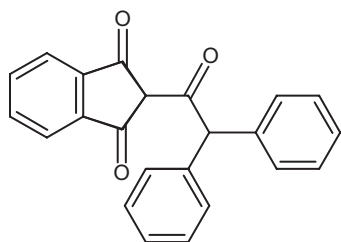
## Indanedione group

### Chlorophacinone



Chlorophacinone (2-(alpha-(4-chlorophenyl)-1-phenylacetyl)-1,3-indandione) was first introduced during the mid-1960s by Liphia S.A. of France at concentrations of 0.05% in baits and 0.2% in tracking dust. Pure chlorophacinone is a yellow crystalline solid that is very soluble in acetone, ethanol, and ethyl acetate but is sparingly soluble in water. It is quite stable, and it is resistant to weathering. Chlorophacinone does not induce "bait shyness" and is compatible with cereals, fruits, roots, and other potential bait substances. Its acute LD<sub>50</sub> in *R. norvegicus* is approximately 20.5 mg/kg, which is less toxic than warfarin, but it has a stronger initial effect on rats and mice. For control of house mice populations, a prolonged feeding period is needed. Chlorophacinone is not effective against warfarin-resistant rodents (Chalermchaikit *et al.*, 1993).

### Diphacinone



Diphacinone (2-diphenylacetyl-1,3-indandione) is an old anticoagulant rodenticide, introduced by Vesicol Chemical Corp. and the Upjohn Company. It has been produced and used primarily in the United States as a 0.005% dry or liquid bait. Pure diphacinone is a yellow powder that is very soluble in chloroform (204 g/kg), toluene (73 g/kg), xylene (50 g/kg), and acetone (29 g/kg) but sparingly soluble in water (0.30 g/L). It will decompose in water due to sunlight. The acute LD<sub>50</sub>s in

*R. norvegicus* are 22.7 mg/kg in females and 43.3 mg/kg in males. It is more toxic than warfarin to rats, mice, and dogs, but its palatability is somewhat lower. Diphacinone is not effective against warfarin-resistant rodents (Chalermchaikit *et al.*, 1993).

The anticoagulant rodenticides are marketed to have efficacy against a number of target pest species. These species are listed next by chemical compounds.

## TARGET SPECIES

### Brodifacoum

One day of feeding on a 0.005% brodifacoum or difenacoum bait was successful in controlling the lesser bandicoot rat (*Bandicota bengalensis*) in Burma (Brooks *et al.*, 1980). Within 3 days, a 0.005% brodifacoum bait gave complete control of golden hamsters (*Mesocricetus auratus*) resistant to 0.005% warfarin and difenacoum (Bradfield and Gill, 1984). A 20-ppm brodifacoum bait gave virtually complete control of Norway rats (*R. norvegicus*) in 21–73 days after other treatments had failed (Greaves *et al.*, 1982). At a 0.005% bait concentration, brodifacoum was more effective at 8 days in controlling *Meriones shawi* than warfarin at 0.025%, coumatetralyl at 0.0375%, difenacoum at 0.005%, and bromadiolone at 0.005% (Gill and Redfern, 1983). The efficacy of brodifacoum has also been shown for *Tatera indica* (Rehman and Ahmad, 1983); *Tatera indica*, *Nesokia indica*, and *Bandicota bengalensis* (Greaves and Rehman, 1977); the field mouse *Mus booduga* Gray (Balasubramanyam *et al.*, 1984); *Rattus rattus* and *Bandicota bengalensis* (Deobhankar, 1985); eight rodent species (Lund, 1981, 1988); the Egyptian spiny mouse (*Acomys cahirinus*) (Mahmoud and Redfern, 1981); and nonresistant (Mosterd and Thijssen, 1991) and warfarin-resistant rats (Rennison and Dubock, 1978). A number of early studies were conducted under the designation WBA 8119 (Rowe and Bradfield, 1976; Rowe *et al.*, 1978). Several different rodenticides have been tested for efficacy against rodent in cucumber (*Cucumis sativus*) plantings (Sabhlok *et al.*, 1997). Brodifacoum has been used to control brown necked ravens (*Corvus ruficollis*) in the Suez Canal region (El-Bahrawy *et al.*, 2007).

### Bromadiolone

Bromadiolone has been effective against warfarin-resistant rats and mice (Redfern and Gill, 1980). It is effective against the Norway rat (*Rattus norvegicus*) (Richards, 1981; Kamil, 1987). Bromadiolone residues have been examined in tissues of *Arvicola terrestris* (Giraudoux *et al.*, 2006) and coypu (*Myocastor coypus*) (Jeantet *et al.*, 1991) after field use. Its effects on the breeding performance of house mice have also been investigated (Twigg and Kay, 1995).

## Chlorophacinone

The efficacy of chlorophacinone against mice, voles, and squirrels has been established. At a 25-ppm bait concentration, chlorophacinone is more effective than coumachlor in controlling common mice (*M. musculus*) in Egypt (Mesban *et al.*, 2003). It can control common voles (*Microtus arvalis*) (Nikodemusz *et al.*, 1981), palm squirrels (*Funambulus pennanti*) (Mathur and Prakash, 1980), and house mice (*M. musculus*) (Lund, 1971).

## Coumafuryl

Coumafuryl is more effective on *R. rattus*, *R. norvegicus*, and *B. bengalensis* than are fumarin and warfarin when used in liquid form (Renapurkar, 1982). It was effective in controlling the cotton rat (*Sigmodon hispidus*) at concentrations used to control *R. rattus* and *R. norvegicus* (Gill and Redfern, 1980) and *Mastomys natalensis* (Gill and Redfern, 1979).

## Coumatetralyl

Median survival time was 4.7 and 11.2 days in *B. bengalensis* and *R. rattus* exposed to 0.0375% coumatetralyl bait (Chopra and Parshad, 1985).

## Diphacinone

Diphacinone has been shown to control rats (Elias and Johns, 1981), vampire bats (Burns and Bullard, 1979, 1980; Thornton, 1980), *B. bengalensis* (Brooks *et al.*, 1980), and coyotes (Szuber and Diechtiar, 1968; Sterner, 1979).

## Difenacoum

Fifty percent of male mice exposed to 0.5mg difenacoum/kg BW died within 9 days, whereas no female mice died (Winn *et al.*, 1989). Norway rats (*R. norvegicus*) fed 25ppm difenacoum bait for 5, 10, or 20 days had whole carcass residues of 0.52–0.74mg/kg BW, with the higher amount being present after the longer feeding period (Rennison and Hadler, 1975; Redfern and Gill, 1980; Atterby *et al.*, 2005). A symptom-dependent taste aversion of the brown rat (*R. norvegicus*) has been reported because the taste aversion is only present when signs are present in the animal (Smith *et al.*, 1994).

## Flocoumafen

Flocoumafen has been demonstrated to control *R. rattus*, *B. bengalensis* (Parshad and Chopra, 1986), *R. norvegicus*

(Buckle, 1986), and the house mouse (*M. musculus*) (Rowe *et al.*, 1985a,b). Flocoumafen gave a quicker and equally effective kill of 68 mice (*M. musculus*) exposed to a 0.005% oatmeal bait compared to difenacoum, brodifacoum, and brodifacoum at the same bait concentrations (Rowe *et al.*, 1985a,b). Nonbiliary intestinal elimination of flocoumafen has been reported in rats (Huckle *et al.*, 1989a,b), and metabolic and toxicological studies have been reported (Veenstra *et al.*, 1991). Accumulation of flocoumafen in rats after repeated exposure has been examined (Huckle *et al.*, 1988).

## Flupropadine

Flupropadine is nearly as effective as calciferol/warfarin in control of the house mouse (*M. musculus*) (Rowe *et al.*, 1985a,b).

## Warfarin

The efficacy of warfarin has been evaluated against squirrels (Chambers and Chambers, 1983) and a host of other species.

## NON-TARGET SPECIES

Unfortunately, non-target species may also be exposed to anticoagulant rodenticides, which are a potential hazard to all species of mammals and birds. The environmental, avian, and wildlife species so exposed are summarized briefly, followed by a discussion of non-target exposure in humans and domestic animals.

## Environmental

Anticoagulant rodenticides may be detected in water, soil, and invertebrates. A method of detecting warfarin in water has been reported (Badia and Diaz-Garcia, 1999), perhaps because anticoagulant rodenticides are used in rice paddies (Baskaran *et al.*, 1995), and accidental discharges of brodifacoum bait may occur in freshwater or marine environments (Primus *et al.*, 2005). The toxicity of anticoagulant rodenticides in soil may be related to the portion not bound to humic acid. Testing for the halogenated biphenyl side chain has been suggested as a way to determine soil exposure to rodenticides (Townsend *et al.*, 1995).

Diphacinone has been detected in snails and slugs in Hawaii. It ranged from 0.8 to 2.5ppm in *Oxychilus* species snails, from 1.3 to 4.0 in *Deroceras laeve* slugs, and up to 1.8ppm in *Limax maximus* slugs (Primus *et al.*, 2006).



## Wildlife

Anticoagulant rodenticides have been detected in polecats and mink in the wild. Difenacoum and bromadiolone were detected in 35% of male (13 of 37) and 38% of female (5 of 13) polecats (*Mustela putorius*) collected in England and Wales in areas where the baits had been used (Shore *et al.*, 2003). Spatial and temporal residues in polecats in Britain have also been reported (Shore *et al.*, 2003). A previous study found difenacoum in 7 of 24 livers (Shore *et al.*, 1996). In France, populations of the free-ranging European mink (*Mustela lutreola*) have declined. Investigators found bromadiolone and chlorophacinone residues in the livers of four species of free-ranging mink and raised the question of the risk to European mink from anticoagulant rodenticides (Fournier-Chambrillon *et al.*, 2004). There are also risks of brodifacoum exposure in non-target birds and animals (Eason *et al.*, 2002). Secondary poisoning of fox after broadcast of anticoagulant rodenticides for voles has been proposed (Raoul *et al.*, 2003). Bromadiolone toxicosis of coypu has also been reported (Morin *et al.*, 1990).

## Avian

Anticoagulant rodenticide toxicosis of birds has been reported throughout the United States and in Australia (Reece *et al.*, 1985). Brodifacoum and difenacoum residues have been detected in a number of non-target avian species. Offspring of turkey vultures (*Cathartes aura*) died after being fed brodifacoum-poisoned mice in a zoo setting. Similar residues have been detected in other carnivorous birds, including *Dacelo novae-guinae* and *Tockus deckeni*.

Ten percent of barn owls collected in England had detectable residues of difenacoum or brodifacoum in their livers. Liver concentrations of difenacoum and brodifacoum ranged from 5 to 106 ng/g and 19 to 515 ng/g, respectively. Mice were fed difenacoum and brodifacoum baits and died within 2–11 days. Poisoned mice were fed to barn owls for 1, 3, or 6 days. All six owls fed difenacoum mice survived, and coagulation times returned to normal in 5–23 days. Four of six owls fed brodifacoum-dosed mice died 6–17 days after feeding. Dead owls had 630–1250 ng/g brodifacoum in their livers (Newton *et al.*, 1990). A white-winged wood duck was treated for brodifacoum toxicity (James *et al.*, 1998), and secondary poisoning of stone martens (*Martes foina*) fed bromadiolone-poisoned mice has been suspected (Lund and Rasmussen, 1986).

Brodifacoum has also been implicated in poisoning of non-target wildlife in 80% of 55 animals investigated in the New York area. Diphacinone, bromadiolone, chlorophacinone, and coumatetralyl were also implicated. Exposure of raptors including great-horned owls (*Bubo virginianus*) and

red-tailed hawks (*Buteo jamaicensis*) constituted half the cases, with gray squirrels (*Scirurus carolinensis*), raccoons (*Procyon lotor*), and white-tailed deer (*Odocoileus virginianus*) constituting the others (Stone *et al.*, 1999).

A compilation of 164 stringiformes collected in western Canada between 1988 and 2003 showed that 70% had residues of at least one rodenticide, and of these, 41% had more than one. Barred owls (*Strix varia*) were most frequently exposed (92%), with the liver concentration of brodifacoum ranging from 0.001 to 0.927 mg/kg and that of bromadiolone ranging from 0.002 to 1.012 mg/kg (Albert *et al.*, 2010).

Broadcast diphacinone has also been implicated in avian toxicoses. Poouli is an endangered avian species in Hawaii. The probability of mortality for adult and juvenile Poouli after a 5-day exposure to broadcast diphacinone has been determined to be 3 and 8%, respectively. Diphacinone residues in snails apparently increase the risk of mortality in juvenile birds (Johnston *et al.*, 2005).

Diphacinone, dicoumarol, and pival have all been shown to increase PT in chicks (Charles *et al.*, 1966). Bromadiolone was detected both in fox (*Vulpes vulpes*) and in buzzards (*Buteo buteo*) in France (Berny *et al.*, 1997).

Chlorophacinone has also been examined. A no-observed-effect concentration of chlorophacinone in feed is 1 mg chlorophacinone/kg feed in Japanese quail (*Coturnix japonica*) (Riedel *et al.*, 1990). Emaciated California quail (*Callopepla californica*) were found to have impacted crops due to parafinized chlorophacinone pellets attributed to the paraffin because PT times were normal (Blus *et al.*, 1985). Chlorophacinone has also been studied in captive kestrels (Radvanyi *et al.*, 1988). A 100% mortality occurred in 1-week-old chicks fed coumestral in wood-straw mats (Munger *et al.*, 1993).

## MECHANISM OF ACTION

The mechanism of action of all anticoagulant rodenticides is similar to that of warfarin – that is, inhibition of vitamin K<sub>1</sub> epoxide reductase (Park *et al.*, 1979; Leck and Park, 1981; Breckenridge *et al.*, 1985). In the coagulation cascade, the clotting factors II, VII, IX, and X must bind calcium ions to be active in clot formation. The Ca<sup>2+</sup> binding ability requires converting glutamyl residues on these clotting factors to  $\gamma$ -carboxyl glutamyl residues by the process of carboxylation. Carboxylation uses vitamin K<sub>1</sub> hydroquinone as a cofactor. This vitamin K-dependent carboxylase reaction converts vitamin K<sub>1</sub> hydroquinone to its epoxide form, vitamin K<sub>1</sub> 2,3-epoxide. In the normal cycle, vitamin K<sub>1</sub> 2,3-epoxide is reduced to the original vitamin K<sub>1</sub> (phyloquinone) by enzyme epoxide reductase and is thus

recycled. Anticoagulant rodenticides produce their effect by interfering with the enzyme vitamin K<sub>1</sub> epoxide reductase, resulting in the depletion of vitamin K<sub>1</sub> and subsequently impairing the synthesis of normal clotting factors II, VII, IX, and X (Craciun *et al.*, 1997, 1998). Clinical coagulopathy soon follows the depletion of vitamin K<sub>1</sub> in the liver. Because these clotting factors in the dog have plasma half-lives of 41, 6.2, 13.9, and 16.5 h, respectively, a lag time of 3–5 days is commonly observed between ingestion of a bait and the onset of clinical signs (Jackson and Suttie, 1977; Suttie, 1986; Murphy and Gerken, 1989). The interrelationship of vitamin K, prothrombin, and  $\gamma$ -carboxyglutamic acid is reviewed in Stenflo (1978). The interaction of warfarin and vitamin K is reviewed in Suttie (1990).

## Biochemistry

Microsomal vitamin K-dependent carboxylase, vitamin K epoxidase, vitamin K<sub>1</sub> epoxide reductase, and cytosolic vitamin K reductase (DT-diaphorase; EC 1.6.99.2) are involved in vitamin K reduction (Hildebrandt and Suttie, 1982). The physiologically important site of action of the anticoagulant rodenticides is vitamin K<sub>1</sub> epoxide reductase (Hildebrandt and Suttie, 1982). Anticoagulant rodenticides act by inhibiting vitamin K<sub>1</sub> 2,3-epoxide reductase and consequently the synthesis of clotting factors II, VII, IX, and X. S-warfarin and difenacoum are more potent in complete inhibition of clotting factor synthesis than are racemic warfarin, R-warfarin, or brodifacoum (Breckenridge *et al.*, 1985).

The greater potency and duration of action of the long-acting or superwarfarins have been attributed to (1) a greater affinity for vitamin K<sub>1</sub> 2,3-epoxide reductase, (2) the ability to inhibit the vitamin K<sub>1</sub> epoxide cycle at more than one point, (3) hepatic accumulation, and (4) unusually long half-lives due to lipid solubility and enterohepatic circulation (Watt *et al.*, 2005). The two diastereomers of brodifacoum may have different conformational alignment with vitamin K epoxide reductase (Cort and Cho, 2009).

Rat liver has two pathways for vitamin K reduction. One is responsible for the therapeutic effect of vitamin K<sub>1</sub> therapy. This pathway is DT-diaphorase and a microsomal dehydrogenase that has 3.6-fold higher activity with NADH than with NADPH. It is not a cytochrome P450 or cytochrome b5 reductase (Wallin, 1986). Although dicoumarol, warfarin, and diphenadione inhibit NAD(P)H in rat liver *in vitro*, only dicoumarol inhibited the enzyme in rats dosed *in vivo* (Schor *et al.*, 1983).

Pathway I is inactive in warfarin and difenacoum intoxicated rats. Vitamin K<sub>1</sub> epoxide reductase was also inactive, so this may be part of pathway I *in vivo*. Pathway II mediates the therapeutic effect of vitamin K<sub>1</sub> and resulting carboxylation *in vitro* (Wallin, 1986).

Vitamin K and vitamin K<sub>1</sub> epoxide can be measured in serum (Bjornsson, 1978; Bjornsson *et al.*, 1979; Donnahey *et al.*, 1979) and tissue. A number of vitamin K detection methods have been reported (Williams *et al.*, 1972; Haroon *et al.*, 1980, 1986, 1987; Haroon and Hauschka, 1983; Hart *et al.*, 1984), including detection in human plasma (Langenberg and Tjaden, 1984) and serum (Lefevre *et al.*, 1979) using high-performance liquid chromatography (HPLC) with electrochemical detection (Takani and Suttie, 1983) or fluorimetric detection in liver (Usui *et al.*, 1989). The mode of action of vitamin K has been reviewed (Olson, 1966).

Because anticoagulant rodenticides inhibit the vitamin K<sub>1</sub> reductase reaction (Pelz *et al.*, 2005), vitamin K<sub>1</sub> epoxide is elevated and vitamin K<sub>1</sub> is reduced. For example, diphenadione- (Mount and Kass, 1989) and warfarin-exposed dogs (Carlisle and Blaschke, 1981) have elevated vitamin K<sub>1</sub> epoxide after subcutaneous vitamin K<sub>1</sub> administration.

Also, vitamin K<sub>1</sub> concentrations are low or nondetectable in rats 24 h after exposure to difenacoum (Winn *et al.*, 1987). Measurement of the epoxide in serum has been proposed as a method to detect surreptitious exposure (Bechtold and Jahnchen, 1979; Bechtold *et al.*, 1983). The disposition of vitamin K in anticoagulant rodenticide poisoning was examined by Park *et al.* (1984).

## Resistance

Resistance to anticoagulant rodenticides is largely transmitted as an autosomal dominant trait. Initially, investigators postulated that a genetic mutation in resistant rodents produced an enzyme epoxide reductase with a reduced binding affinity for warfarin and similar anticoagulants that resulted in resistance to warfarin (Bell and Caldwell, 1973; Zimmerman and Matschiner, 1974; Hadler and Shadbolt, 1975; Misenheimer *et al.*, 1994). This theory seems to have been borne out.

Eight different mutations in the vitamin K reductase reaction have been identified in laboratory strains of brown rats and house mice and in wild-caught brown rats. Five mutations are of only two amino acids. Resistance to warfarin is largely influenced by mutations at Tyr139 (Pelz *et al.*, 2005). The genomic assignment of the warfarin-resistant locus (Kohn and Pelz, 1999) and a gene anchored map of the rat warfarin-resistant locus (Kohn and Pelz, 2000) have been reported. These followed reports of the biochemical basis of warfarin and bromadiolone resistance in house mice (Misenheimer *et al.*, 1994). However, warfarin-resistant rats are not resistant to coumatetralyl, so there may be more than one mechanism of resistance (Bell *et al.*, 1976).

Feeding of difenacoum and menadione (vitamin K<sub>3</sub>) for 4 days is used as a test to determine the degree

of resistance of laboratory Norway rats (*R. norvegicus*) and wild rats to difenacoum (Gill *et al.*, 1993). Chemical differences of the side chains at the 3-position of the second-generation 4-hydroxycoumarins may provide a point of attachment to a lipophilic site on the epoxide reductase enzyme, where warfarin and other first-generation rodenticides do not bind strongly (Hadler and Shadbolt, 1975). This increased binding increases their toxicity and may reduce the development of resistance in rodents.

An absolute requirement for vitamin K<sub>1</sub> in Danish Norway rats (*R. norvegicus*) (Markussen *et al.*, 2003) and others resistant to warfarin remains is another theory (Trivedi *et al.*, 1988). Amino acid substitutions of the vitamin K epoxide reductase complex subunit 1 and increased expression of P450 (CYP) have been postulated (Ishizuka *et al.*, 2008).

## Antioxidant

The anticoagulant rodenticides and oral anticoagulants are also of interest in cancer research. In part, because catalyzing obligatory two-electron reductions of quinones to hydroquinones, NAD(P)H: quinone reductase (QR1) protects cells against the deleterious effects of redox cycling of quinones and their ability to deplete glutathione and produce neoplasia (Dinkova and Talalay, 2000). DT-diaphorase and coenzyme Q appear to have antioxidant and pro-oxidant functions in quinone metabolism (Beyer, 1994; Cadenas, 1995).

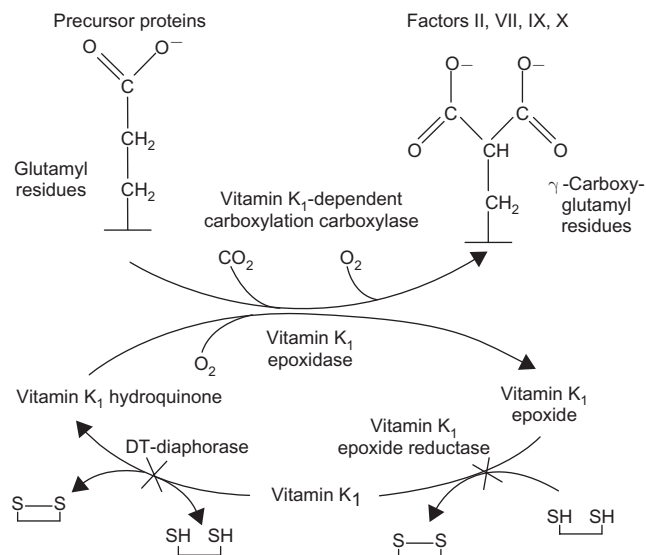
## Metabolism

Warfarin is metabolized by CYP 2C9 and 2C19 in humans (Goldstein, 2001; Brandon *et al.*, 2005). The CYP 2C9 enzyme has several inherited polymorphisms (Kirchheiner and Brockmoller, 2005). Chlorophacinone elimination may be enhanced by phenobarbital administration (Burocoa *et al.*, 1989; Lagrange *et al.*, 1999), perhaps due to CYP induction. The effect of phenobarbitone on vitamin K<sub>1</sub> metabolism (Wilson and Park, 1984) can be compared to that of the rabbit (Winn *et al.*, 1988).

# TOXICOKINETICS

## Bioavailability

Most anticoagulant rodenticide toxicoses occur after oral exposure. However, a diphacinone-induced coagulopathy has been reported after dermal exposure to a liquid preparation (Spiller *et al.*, 2003). A quite unusual case



**FIGURE 56.1** Liver metabolism of vitamin K. The dithiol-dependent vitamin K<sub>1</sub> epoxide reduction and vitamin K<sub>1</sub> (phyloquinone) reduction are the metabolic steps inhibited by anticoagulant rodenticides.

is exposure to brodifacoum after donation and transplantation of multiple organs (Ornstein *et al.*, 1999).

The oral bioavailability of warfarin, chlorophacinone, and bromadiolone was estimated to be 79, 92, and 88%, respectively, in sheep. These anticoagulants degraded by approximately 15% over 24h in rumen extracts (Berny *et al.*, 1995). The bioavailability of warfarin is influenced by dietary protein (Barber and Colvin, 1980).

## Distribution

Sixty percent of <sup>14</sup>C-labeled diphacinone is eliminated in feces and 10% in urine over 4 days in mice and 8 days in rats (Yu *et al.*, 1982). Tissue distribution indicated that liver had the most <sup>14</sup>C activity, with the lowest amounts in brain, muscle, blood, and fat (Yu *et al.*, 1982). The disposition and pharmacodynamic properties of brodifacoum have also been characterized in rats (Bachmann and Sullivan, 1983). Similarly, 30% of <sup>14</sup>C-labeled fluocoumafen is eliminated in feces and less than 3% in urine within 3 days in rats (Huckle *et al.*, 1988). Approximately 60% of <sup>14</sup>C fluocoumafen is liable to  $\beta$ -glucuronidase, and most radioactivity is found unchanged in the liver. Elimination is biphasic, with a rapid phase of 5 days and then a prolonged phase of 100 days in Japanese quail (Huckle *et al.*, 1989a,b).

Alteration of parental behavior was speculated to be a cause of 54% lethality in offspring in the face of 40% lethality of lactating female albino rats dosed with coumatetralyl (Marchini and Turillazzi, 1978). However, passage into milk is an alternative theory.

## Elimination

The different chemical structures give different elimination kinetics for the various anticoagulant rodenticides. Elimination kinetics is estimated from human or animal clinical cases in many instances. Such cases are presented for the 4-hydroxycoumarins, warfarin, brodifacoum, difenacoum, bromadiolone, difethialone, and chlorophacinone.

Warfarin has a terminal half-life of  $5.6 \pm 0.7$  h with a monoexponential decay (Breckenridge *et al.*, 1985). Brodifacoum and difenacoum have a biexponential decay of  $60 \pm 1.9$  and  $83 \pm 10$  h, respectively, in rabbits (Breckenridge *et al.*, 1985). Although a median half-life of brodifacoum elimination has been estimated to be 2.4 days in seven dogs (Robben *et al.*, 1998), the data may reflect the first elimination phase.

A 41-year-old man had a terminal half-life of 11.7 days for difenacoum after covert administration of an unknown amount of the drug (McCarthy *et al.*, 1997). A second individual had a peak serum concentration of 600 ng/ml of difenacoum (Butcher *et al.*, 1992).

A peak serum concentration of 440  $\mu$ g/L and an elimination half-life of 140 h were reported in a 55-year-old man who ate an unknown amount of bromadiolone (Grobosch *et al.*, 2006). Elimination half-lives of 2.2 and 3.2 days have been reported in two dogs with difethialone exposure (Robben *et al.*, 1998). A half-life of 5 days has been reported in sheep dosed with pindone (Robinson *et al.*, 2005).

## Duration

Despite reported elimination half-lives, the duration of anticoagulant effect provides an indication of the clinically relevant treatment times. Brodifacoum and difenacoum cases seem to have the longest duration of anticoagulant effect in animals and humans.

Rabbits are anticoagulated 6 weeks after oral exposure to 1 mg/kg BW brodifacoum (Park and Leck, 1982). Although clotting times were prolonged for 7 months after a person's exposure to an unknown amount of difenacoum (McCarthy *et al.*, 1997), elevated concentrations of vitamin K<sub>1</sub> 2,3-epoxide were detected for 18 months after exposure of two factory workers to brodifacoum and difenacoum despite normal prothrombin times (Park *et al.*, 1986).

Duration of treatment has also been reported for bromadiolone and chlorophacinone cases and for pindone-dosed sheep. A patient exposed to bromadiolone had to be treated for 6 months (Haug *et al.*, 1992). An 18-year-old woman's PT normalized 7 weeks after deliberate ingestion of 100 mg chlorophacinone (Vogel *et al.*, 1988). Pindone has been detected for up to 14 days in blood, 17 days in fat, and 39 days in liver of sheep dosed with it (Robinson, 2005).

## TOXICITY

### Occurrence

Anticoagulant rodenticides comprise the most common rodenticide exposure of dogs and the most common toxins seen in many U.S. veterinary practices (Beasley and Trammel, 1989; Murphy *et al.*, 1989). Dogs suspected of anticoagulant rodenticide poisoning in the Netherlands had brodifacoum (19), bromadiolone (14), difenacoum (8), difethialone, and (6) chlorophacinone (1) (Robben *et al.*, 1997). Assessment of potential toxicity of pindone for domestic animals has also been performed (Martin *et al.*, 1991).

### Dose

A 25-year-old man attempted suicide by eating four 42-g boxes of 0.005% brodifacoum bait and succeeded in developing a coagulopathy (Kruse and Carlson, 1992). A 33-year-old man ate 1875 mg of chlorophacinone (Lagrange *et al.*, 1999), and an 18-year-old female deliberately ingested 100 mg chlorophacinone (Vogel *et al.*, 1988) and became anticoagulated. Sheep dosed orally with 10, 3, or 2 mg/kg BW pindone also developed coagulopathies (Robinson *et al.*, 2005). Coumatetralyl poisoning has been reported in pigs (Dobson, 1973) and pest species (Rowe and Redfern, 1968; Greaves and Ayres, 1969).

Rodenticides may be more toxic when repeatedly ingested over several days than when an equal amount is consumed in a single feeding (Dorman, 1990). Susceptibility may be greater in hypoprothrombinemic juveniles or animals with malabsorption syndromes. Also, the concurrent administration of highly protein-bound drugs (e.g., phenylbutazone and aspirin) or disease states, such as chronic renal disease, may increase the susceptibility of individuals to anticoagulant rodenticide poisoning (Beasley and Buck, 1983; Mount *et al.*, 1985). Transdermal exposure is a rare but not unprecedented route of exposure for toxicosis (Binks and Davies, 2007).

## DIAGNOSIS

### History

A clinical diagnosis of anticoagulant rodenticide poisoning is most often dependent on a history of exposure or clinical signs, evidence of a coagulopathy, and response to vitamin K<sub>1</sub> therapy. The most pragmatic approach for determining the specific anticoagulant rodenticide involved is to read the product package. This approach alone is not definitive because as many as 25% of



anticoagulant rodenticide-intoxicated dogs do not have the anticoagulant from the serum the owners suspect (Murphy and Gerken, 1989).

Most anticoagulant rodenticide toxicoses is accidental; however, some is intentional (Mack, 1994) in both humans and animals (Misra *et al.*, 2010). For example, in the United States in 2004, 16,054 cases of exposure were reported as accidental ingestions by children; however, 4576 cases required hospitalization, 23 had major adverse outcomes, and there was one death (Binks and Davies, 2007).

Intent may become a legal issue in malicious poisoning cases. Spontaneous hemorrhage after accidental brodifacoum exposure in a child is known to rarely occur (Watts *et al.*, 1990; Travis *et al.*, 1993; Beriain Rodríguez *et al.*, 2008). Iatrogenic cases in adults occur with more frequency (Weitzel *et al.*, 1990; Waijen *et al.*, 2001; Walker and Beach, 2002; Moery and Pontious, 2009; Hong *et al.*, 2010), and combinations of anticoagulant rodenticides and glass (Tsutaoka *et al.*, 2003) or ethylene glycol (Seidemann *et al.*, 1995) also occur. As many as three different compounds have been detected in one woman (Zolcinski *et al.*, 2008). Human bromadiolone toxicosis has been reported in China (Shi *et al.*, 2005) and is known elsewhere (Shanberge, 1988). Consequently, a history of exposure or lack thereof is not always present or reliable.

## Clinical signs

A history of exposure to anticoagulant rodenticides is not always available, so the toxicity must be inferred from the clinical signs. Clinical signs have been reported in a number of human and animal cases. Humans have dosed themselves or their children (Babcock *et al.*, 1993). Anticoagulant rodenticides have been added to potentiate drugs of abuse (Spahr *et al.*, 2007). Most are related to the coagulopathy. Animals exposed to toxic doses of anticoagulant rodenticides remain asymptomatic until depletion of the active clotting factors occurs. Therefore, clinical signs generally do not develop until 1 or 2 days (Dorman, 1990) or 3–5 days (Murphy and Gerken, 1989) postingestion.

A 55-year-old man had red sputum after ingesting an unknown amount of bromadiolone (Groboch *et al.*, 2006). Epistaxis, gingival bleeding, widespread bruising, hematomas, hematuria, menorrhea, gastrointestinal bleeding, rectal bleeding, hemorrhage into all body organs, and anemia have been reported (Watt *et al.*, 2005). A 36-year-old woman had abdominal pain, hematuria, and red blood in her stool with diffuse cutaneous hematomas after ingesting difenacoum for several weeks. She had hemoperitoneum, urethral hematoma on tomography (Barlow *et al.*, 1982; Berry *et al.*, 2000; Terneu *et al.*, 2003), and diffuse alveolar hemorrhage (Barnett *et al.*, 1992). Hemoperitoneum after fluconazole

administration has been reported in a brodifacoum exposure case (Kim *et al.*, 2010). Hematuria was reported in eight of nine patients who ingested superwarfarins (Nelson *et al.*, 2006; Wu *et al.*, 2009). A case of hemarthrosis has also been reported (Kotsaftis *et al.*, 2007).

A 51-year-old woman who later admitted chronic ingestion of difenacoum was admitted with hemoperitoneum and intramural hematoma of the small intestine (Soubiron *et al.*, 2000). In another case, epistaxis, hematoma, purpura and bruising were observed in a 41-year-old man with difenacoum (McCarthy *et al.*, 1997; Laposata *et al.*, 2007). Frank hematuria (Butcher *et al.*, 1992), neck pain, and cervical hematoma have also been observed in humans with diphenacoum exposure (Nighoghossian *et al.*, 1990). Acquired coagulopathy has been reported due to anticoagulant rodenticide exposure (Humphry, 1989; Huic *et al.*, 2002).

Human pleural, pericardial, mediastinal, and sub-arachnoid hemorrhages are reported with brodifacoum exposure (Kruse and Carlson, 1992), and gastric and pulmonary hemorrhage (Olmos and López, 2007) and hematemesis have been reported (Dolin *et al.*, 2006). Cases of chlorophacinone poisoning in humans have also been reported (Murdoch, 1983; Dusein *et al.*, 1984). Hemoperitoneum from brodifacoum overdose has been observed (Morgan *et al.*, 1996; Kim *et al.*, 2010). Gastrointestinal hemorrhage has been reported after endoscopic cold mucosal biopsy (Zhao *et al.*, 2010). A case of intestinal obstruction associated with superwarfarin poisoning has been reported (Nie *et al.*, 2010). Bruising, hematuria, and abdominal pain secondary to a perinephric hematoma have also been reported (Kapadia and Bona, 2008). A number of other cases of brodifacoum (Braithwaite, 1982; Corke, 1997; Stanziale *et al.*, 1997; Casner, 1998; Bruno *et al.*, 2000), bromadiolone (Chow *et al.*, 1992), and other anticoagulant rodenticide exposure are present in the human literature (Ross *et al.*, 1992).

Clinical signs in animals are largely from canine cases (Woody *et al.*, 1992). Sometimes the only clinical signs in anticoagulant poisoned animals are dyspnea, lethargy, or anorexia, but more often depression, weakness, pallor, and ventral hematomas are present (DuVall *et al.*, 1989). In addition, pulmonary edema, pleural effusion, pericardial effusion (Schulman *et al.*, 1986), intratracheal hemorrhage (McGuire *et al.*, 1999), thymic hemorrhage (Elsinghorst, 2003), laryngeal obstruction (Peterson and Streeter, 1996), pericardial effusion and cardiac tamponade (Petrus and Henik 1999), renal subcapsular hemorrhage (Radi and Thompson, 2004), and hematometra (Padgett *et al.*, 1998) have been reported.

Additional cases of diphacinone (Schulman *et al.*, 1986; Troy, 1988) and brodifacoum toxicosis (Grayson, 1982; McSporran and Phillips, 1983; Booth, 1989; Baker *et al.*, 2002) in dogs are reported. Horses have been exposed to these rodenticides experimentally (Boermans *et al.*, 1991)

and in the field (McConnico, 1997; Ayala *et al.*, 2007). Brodifacoum may have been observed in neonatal puppies (Munday and Thompson, 2003) and has been successfully treated in a pregnant bitch (Hornfeldt and Phearman, 1996).

In clinical settings, prolonged bleeding from injection sites is usually noticed. A few clinical signs are reported that are not directly attributable to the coagulopathy. A paradoxical venous thrombosis was reported in a person with chlorophacinone exposure (Papin *et al.*, 2007). Death, reduced breeding performance, stillborn and non-viable lambs, as well as reduced sperm motility of rams are reported in sheep dosed with pindone (Robinson *et al.*, 2005).

## Coagulopathy

Evidence of a coagulopathy is the second element of the diagnosis. Coagulation tests are normally run on live animals, and a necropsy is performed on dead animals to support the presence of a coagulopathy. The basic mechanisms of clotting have been reviewed (Seegers, 1969).

## Hematology

The anticoagulant rodenticides reduce activity of factors II, VII, IX, and X in circulation. For example, activities of 5% for factor II, 8% for factor VII, 4% for factor IX, and 6% for factor X have been reported in a brodifacoum case (Wu *et al.*, 2009; Kim *et al.*, 2010). The one-stage prothrombin time (OSPT) for evaluating factor VII is the most sensitive tool for early diagnosis because factor VII has the shortest half-life of the vitamin K<sub>1</sub>-dependent clotting factors – approximately 6.2h in dogs. Activated partial thromboplastin time (aPTT) tests for all coagulation factors except factor VII are usually used in conjunction with OSPT. Activated coagulation time (ACT) is used in the same way as aPTT. ACT is easiest to use in a clinic setting because it requires only diatomaceous earth tubes and a heater block or water bath (Byrne, 1970).

Laboratory test results of abnormal prolonged OSPT, aPTT, and ACT in the presence of normal thrombin time (TT), fibrinogen, circulating fibrin degradation products (FDPs), and platelet counts are consistent with anticoagulant rodenticide poisoning. However, animals with severe anemia may have elevated FDPs and reduced platelet counts. An elevated INR is frequently measured in human coagulopathy cases (Schmeits *et al.*, 2009; Boettcher *et al.*, 2011). Thrombosis is rarely reported (Papin *et al.*, 2007; De Paula *et al.*, 2009).

The diagnostic protocol based on these coagulation factor evaluation tests (OSPT, aPTT, and ACT) cannot differentiate between short- and long-acting anticoagulant rodenticide poisoning. The ability to recognize long-acting anticoagulants is critical because therapeutic

success may be based on the duration of vitamin K<sub>1</sub> treatment. The ability to identify the specific anticoagulant rodenticide involved using analytical chemistry is discussed later.

Coagulation testing is not always indicated after minor exposure. Of 110 children who ingested anticoagulant rodenticides, 8 had prolonged PTs. Seventeen percent (6 of 34) had prolonged PTs 48h after exposure, whereas only 1.9% (2 of 104) had prolonged PTs 24h after exposure. It is recommended to check PTs 24 and 48h after exposure in children (Smolinske *et al.*, 1989). Routine measurement of the INR is unnecessary in young children because the amounts ingested are invariably small (Watt *et al.*, 2005).

Although some bias in pediatric brodifacoum exposure data is suspected (Osterhoudt and Henretig, 2003), prospective studies of acute, unintentional, pediatric superwarfarin ingestions managed without (Ingels *et al.*, 2002) or with (Smolinske *et al.*, 1989) decontamination have been conducted. Some argue against the need for a PT in unintentional pediatric superwarfarin exposures (Mullins *et al.*, 2000; see also cases of acute pediatric brodifacoum ingestions (Shepherd *et al.*, 2002)). Although less treatment is often better for accidental pediatric brodifacoum exposures (Kanabar and Volans, 2002), some pediatric cases have coagulopathies despite early treatment (Montanio *et al.*, 1993).

In all other cases, the INR should be measured 36–48h postexposure. If the INR is normal at this time, even in the case of long-acting formulations, no further action is required (Watt *et al.*, 2005).

The most significant elevation of PT, PTT, and protein induced by vitamin K absence (PIVKA) was observed 72h after a single dose of diphacinone of 2mg/kg in ground squirrels (*Spermophilus beecheyi*), although elevations were seen at 24h (Whisson and Salmon, 2002). Increases in ACT, aPTT, and OSPT are often used to support a clinical diagnosis of anticoagulant rodenticide poisoning in dogs (Dorman, 1990). Coagulation factor synthesis may be inhibited for up to 30 days in diphacinone-exposed dogs (Mount and Feldman, 1983).

Prothrombin time should be checked 48h after stopping vitamin K<sub>1</sub> therapy to detect any recurrence of coagulopathy (Chataigner *et al.*, 1989). PTs in sheep were stable in samples stored at 0°, 20°, and 30° for 24h (Shlosberg and Egyed, 1985).

Vitamin K-dependent factor activity has been suggested for rapid identification of surreptitious brodifacoum poisoning (Miller *et al.*, 2006).

## Necropsy/autopsy

Pleural, pericardial, mediastinal, and subarachnoid hemorrhages have been reported in humans with

brodifacoum exposure (Kruse and Carlson, 1992; Tahir *et al.*, 2008).

Hemoperitoneum, hemothorax, and pulmonary hemorrhage are the most common necropsy findings in dogs and cats with anticoagulant rodenticide residues in liver (DuVall *et al.*, 1989; Rickman and Gurfield, 2009). Pulmonary edema, pleural effusion, pericardial effusion (Schulman *et al.*, 1986), and intratracheal hemorrhage are reported as well (McGuire *et al.*, 1999).

Many cases of anticoagulant poisoning are subacute in nature, but sudden death may occur as the result of acute internal hemorrhage. Evidence of external hemorrhage, such as melena, epistaxis, hematemesis, hematuria, gingival bleeding, or excessive bleeding from an open wound, may or may not be seen. However, internal hemorrhage involving the lungs, pleural and/or peritoneal cavities, and facial planes is commonly reported (DuVall *et al.*, 1989; Murphy and Gerken, 1989). Massive tracheal and esophageal hemorrhage 2 days after bait ingestion has been reported in a dog (Stowe *et al.*, 1983). In New Zealand, where brodifacoum was used in wild rabbit population control, 43 rabbit carcasses were found with massive hemorrhage in the abdominal cavity (52%) and thoracic cavity (17%), and the remaining 31% of cases were found with hemorrhage of muscles, cecum, stomach, kidney, mesentery, and placenta of pregnant does (Rammell *et al.*, 1984). Postmortem findings in warfarin poisoning have been described by Dakin (1968).

#### Response to vitamin K<sub>1</sub> treatment

Remission of the coagulopathy 24h after vitamin K<sub>1</sub> treatment supports a clinical diagnosis of a vitamin K<sub>1</sub>-responsive coagulopathy (Tvedten, 1989). A clinical diagnosis is not sufficient in all cases. Some cases require that an etiological diagnosis be made. Analytical chemistry testing is required in such cases.

#### Analytical chemistry

A number of analytical methods have been reported for detecting anticoagulant rodenticides in various matrices. Fluorimetric and gas chromatography methods have been commonly used to detect warfarin in serum (O'Reilly *et al.*, 1962; Corn and Berberich, 1967; Lewis *et al.*, 1970; Welling *et al.*, 1970; Mildha *et al.*, 1974; Vessell and Shivley, 1974; Fasco *et al.*, 1977; Hanna *et al.*, 1978; Lee *et al.*, 1981).

Warfarin-specific methods were generally not adequate for the anticoagulant rodenticides, so a number of other methods were developed, including thin-layer chromatography (TLC), HPLC, mass spectroscopy (MS), and antibody-mediated tests. Coumarin

anticoagulant rodenticides were initially detected using TLC (Lau-Cam and Chu-Fong, 1972; Mallet *et al.*, 1973). A high-performance TLC method with an estimated detection limit of 200 ppb and 87% recovery from liver has been reported (Berny *et al.*, 1995).

Early HPLC methods were focused on an individual chemical. For example, methods to detect chlorophacinone in formulations (Grant and Pike, 1979; Gy *et al.*, 1981), brodifacoum in serum (Murphy and Gerken, 1989), brodifacoum (Koubek *et al.*, 1979; Keiboom and Rammell, 1981; Hoogenboom and Rammell, 1983; Ray *et al.*, 1989; Hong *et al.*, 2010; Kim *et al.*, 2010), bromadiolone (Hunter *et al.*, 1988; Subbiah *et al.*, 2005), chlorophacinone (Hunter, 1984), difethiolone (Goldade *et al.*, 1998), and difenacoum (Mundy and Machin, 1977) in tissue have been reported. Then a method was developed to search for all the anticoagulant rodenticides on the market at the same time. It succeeded in extracting and detecting eight anticoagulant rodenticides in serum and liver using fluorescence and ultraviolet (UV) detection. Samples are extracted with acetonitrile and then cleaned up on solid-phase columns. Four hydroxycoumarins are detected by fluorescence with excitation at 318 nm and emission at 390 nm. The indanediones are detected at 285 nm. An extraction recovery of 75% for serum and 69% from liver was reported. Hydroxycoumarins may be detected down to approximately 1 ng/ml of serum and 1 ng/g of liver, and indanediones may be detected down to 10 ng/ml of serum and 10 ng/g of liver (Felice and Murphy, 1989; Felice *et al.*, 1991; Chalermchaikit *et al.*, 1993). Another HPLC method for detecting brodifacoum in serum and liver using difenacoum as the internal standard has been reported (O'Bryan and Constable, 1991). A method for the simultaneous detection of five superwarfarin rodenticides in human serum has been described by Kuijpers *et al.* (1995). Other serum methods have been reported with detection limits of 3–12 ng/ml for fluorescence and 20–75 ng/ml for UV detection (Mura *et al.*, 1992; Kuijpers *et al.*, 1995; McCarthy *et al.*, 1997; Feng *et al.*, 1999; Jin *et al.*, 2007).

Tissue methods include a solid-phase cartridge extraction from liver with recoveries ranging from 52% for difenacoum to 78% for warfarin. The limit of detection is 10 ppb for warfarin and difenacoum and 110 ppb for chlorophacinone (Addison, 1982; Jones, 1996; Fauconnet *et al.*, 1997).

HPLC methods have also been published to distinguish *cis* and *trans* isomers of difenacoum with detection limits of 5 ng/ml (Kelly *et al.*, 1993). An early interesting approach was the use of a post-column pH shift to enhance fluorescence detection (Hunter, 1985; Hunter *et al.*, 1988). Several HPLC methods have also been reported (AOAC, 1976a,b; Mundy and Machin, 1982; Hunter *et al.*, 1983a,b) for diphacinone (Bullard *et al.*, 1975, 1976) with fluorescence detector for bromadiolone



(Deepa and Mishra, 2005), brodifacoum (Hagenboom and Rammell, 1983; Fu *et al.*, 2006), and difenacoum (Hadler and Shadbolt, 1975) and for determination of Rozol in parafinized formulations (Kawano and Chang, 1980) and bromadiolone in tissues (Nahas, 1986).

Another method uses diode array detector (Yang *et al.*, 2001). An interesting method uses HPLC to detect anticoagulant rodenticides in soft drinks (Dimuccio *et al.*, 1991). Also, an ion pair liquid chromatography method has been reported to detect chlorophacinone and diphacinone in oats (Primus *et al.*, 1998).

Contemporary confirmatory methods use mass spectrometry. Liquid chromatography–electrospray ionization–mass spectroscopy (LC-ESI-MS) has been reported for the analysis of 10 anticoagulant rodenticides with a limit of quantitation of approximately 5 µg/L (Grobosch *et al.*, 2006; Jin and Chen, 2006; Jin *et al.*, 2007). Other methods use LC-MS-MS for drugs including warfarin (Marquet *et al.*, 2003) and flocoumafen (Boettcher *et al.*, 2011) and use LC-ESI-MS and HPLC-UV to detect anticoagulant rodenticides as low as 20 ng (Mesmer and Flurer, 2000). One of the earlier MS methods used a direct probe technique to detect indanedione residues in food animals (Braselton *et al.*, 1992).

A cell culture/enzyme-linked immunosorbent assay has been developed to detect anticoagulant rodenticides in treated grain (Lawley *et al.*, 2006). A prior immunoassay was developed to detect diphacinone and chlorophacinone (Mount *et al.*, 1988). Enantiomers of warfarin, coumachlor, and coumafuryl can be separated chromatographically (Armstrong *et al.*, 1993).

The serum concentration in dogs with anticoagulant rodenticide poisoning ranges from 10 to 851 ng/L for brodifacoum, difethialone, and difenacoum (Robben *et al.*, 1998).

## TREATMENT

General, supportive, and specific treatments are available for anticoagulant rodenticide toxicosis. A prospective study of the outcome of patients with excessive warfarin exposure is described by Hylek *et al.* (2000).

### General

Emetic, adsorbent, and cathartic therapies are indicated if the ingestion of the anticoagulant rodenticide has occurred within the past few hours. Peak serum concentrations of brodifacoum occur 2 h after oral dosing, however, so the coagulation status of the animal should be monitored at 24 and 48 h after exposure.

Clinical coagulopathy normally occurs 2–5 days after oral exposure, so emetics and cathartics at the time of presentation are not normally indicated. Oral-activated charcoal therapy, however, may be useful for those chemicals that undergo enterohepatic circulation.

### Supportive

Recommendations for humans with anticoagulant rodenticide toxicosis have been reported by Watt *et al.* (2005). If active bleeding occurs, 50 units/kg prothrombin complex concentrate (which contains factors II, VII, IX, and X) or 1.2–4.8 mg recombinant activated factor VII, or 15 mL/kg fresh frozen plasma if no concentrate is available, and 10 mg phytonadione intravenously (100 µg/kg BW in a child) should be given. If there is no active bleeding and the INR is 4.0 or less, no treatment is required; if the INR is greater than 4.0, 10 mg phytonadione should be administered intravenously (Watt *et al.*, 2005).

Animals with severe clinical bleeding or markedly reduced PCVs should receive fresh plasma or blood transfusions because of the 4–8 h often required to increase clotting factor activity following vitamin K<sub>1</sub> therapy (Chalermchaikit *et al.*, 1993). These recommendations are consistent with those of other studies (McCarthy *et al.*, 1997; Soubiron *et al.*, 2000; Terneu *et al.*, 2003).

### Specific

Vitamin K<sub>1</sub> therapy is recommended in humans (McCarthy *et al.*, 1997; Soubiron *et al.*, 2000; Terneu *et al.*, 2003; Boettcher *et al.*, 2011) and animals with elevated coagulation times after exposure to anticoagulant rodenticides (Mount *et al.*, 1982; Murphy *et al.*, 1989; Robben *et al.*, 1998). Vitamin K<sub>1</sub> (phyloquinone) is the most effective form for the treatment of anticoagulant rodenticide intoxication because of its immediate availability for the synthesis of new clotting factors (Chalermchaikit *et al.*, 1993).

The pharmacological half-life of vitamin K<sub>1</sub> is  $1.7 \pm 0.1$  h in rabbits dosed with brodifacoum (Park and Leck, 1982). Prothrombin activity reaches peak improvement 4 h after administration of vitamin K<sub>1</sub> to rabbits anticoagulated with brodifacoum or difenacoum (Park and Leck, 1982).

The duration of vitamin K<sub>1</sub> treatment is prolonged for the longer acting chemicals (Butcher *et al.*, 1992). It may be required for up to 2 weeks in diphacinone-exposed dogs (Mount and Feldmam, 1983). Daily doses of vitamin K<sub>1</sub> in the range of 0.25–2.5 mg/kg for 1 week are recommended for exposure to short-acting rodenticides, and doses of 2.5–5.0 mg/kg for 3 or 4 weeks are



recommended for exposure to long-acting rodenticides (Mount *et al.*, 1985). Prolonged anticoagulation in rat poisoning has been reported by Jones *et al.* (1984) and Lipton and Klass (1984). Treatment for 48 days has been reported in a human case of flocoumafen exposure in which a half-life of 6.7 days was estimated (Boettcher *et al.*, 2011). In intentional poisoning cases in humans, vitamin K<sub>1</sub> treatment may be required for 2 months (Dolin *et al.*, 2006) or 3 months (Wu *et al.*, 2009). A brodifacoum case had a 56-day half-life (Olmos and López, 2007).

The two most commonly recommended routes of vitamin K<sub>1</sub> administration are oral and subcutaneous. Intramuscular injections in a hypoprothrombinemic animal can produce hematoma, and intravenous administration of vitamin K<sub>1</sub> has been associated with anaphylaxis; therefore, these routes are discouraged in the therapeutic regimen (Clark and Halliwell, 1963).

Vitamin K<sub>1</sub> therapy may be reduced in a stepwise manner as long as the PT remains normal (Robben *et al.*, 1998). The length of treatment is currently determined by evaluation of OSPT values for 2 days after the cessation of vitamin K<sub>1</sub> administration. If prolonged OPST is found, treatment is commonly continued for another week, but if it remains normal for 5 or 6 days, the vitamin K<sub>1</sub> treatment is usually terminated (Murphy *et al.*, 1989).

A diagnostic approach to the bleeding patient has been described by Johnstone (1989). A diagnostic protocol should utilize more than one coagulation test because it is necessary to differentiate rodenticide poisoning from other coagulopathies, such as disseminated intravascular coagulopathy, congenital factor deficiencies, hyperviscosity syndromes, platelet deficiencies or functional defects, von Willebrand's disease, and canine ehrlichiosis. Hypovitaminosis K associated bleeding has been reported in cats with malabsorption syndrome (Edwards and Russell, 1987).

## CONCLUSIONS

Warfarin was the first anticoagulant rodenticide introduced into the market soon after World War II and was widely used in many countries. Many other anticoagulant rodenticides with similar potency to that of warfarin were also synthesized (LD<sub>50</sub>s ranging from 10 to 50 mg/kg BW). These compounds were recognized as the first-generation anticoagulant rodenticides. Due to the problem of tolerance development in rodents, newer compounds with a greater toxicity (acute LD<sub>50</sub>s of 0.2–3.9 mg/kg) were developed. These compounds are called second-generation anticoagulant rodenticides or superwarfarins. Based on chemical structures,

anticoagulant rodenticides are classified into two categories: hydroxycoumarines and indanediones. Common examples of the former group are brodifacoum, bromadiolone, coumatetralyl, difenacoum, and warfarin, and examples of the latter group are chlorofacinone and diphacinone. Whereas intentional poisoning is common in dogs, secondary poisoning is common in avian and wildlife species. The mechanism of action of all anticoagulant rodenticides is similar to that of warfarin – that is, inhibition of vitamin K<sub>1</sub> epoxide reductase, resulting in the depletion of vitamin K<sub>1</sub>. Subsequently, this impairs the synthesis of normal clotting factors II, VII, IX, and X, and ultimately coagulopathy ensues due to depletion of vitamin K<sub>1</sub> in the liver. Diagnosis is based on history of rodenticide exposure, clinical signs of hemorrhage and coagulopathy, and chemical confirmation in body tissues or fluids. Treatment includes vitamin K<sub>1</sub> and blood transfusion.

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## Non-anticoagulant rodenticides

Ramesh C. Gupta

### STRYCHNINE

#### Introduction

Strychnine is an alkaloid derived from the seeds and bark of a tree, *Strychnos nux-vomica*, that is native to Southeast Asia (India, West Indies, Sri Lanka, and Indonesia) and Australia. Strychnine is also found in *Strychnos ignatii*. Strychnine is a white, odorless, crystalline powder, which has the chemical formula  $C_{21}H_{22}N_2O_2$  and a molecular weight of 334.41. Its structural formula is shown in Figure 57.1.

Nux-vomica/strychnine has been used for at least five centuries for both pests and people. Its major use is as a pesticide (rodenticide, avicide, and insecticide), but it is also used as a therapeutic agent in human ailments (laxative, appetizer, and central nervous system (CNS) stimulant). In addition, strychnine in small amounts is known to be added to LSD, heroin, cocaine, and other "street drugs," and it has been known to cause poisoning (O'Callaghan *et al.*, 1982). In the 1960s, strychnine was the title of a song by the rock band "The Sonics"; the song includes the lines, "Some folks like the water, some folks like the wine; I like the taste of straight strychnine." Strychnine has many names and is sold under various trade names, including Boomer-Rid, Certox, Dog-button, Dolco mouse Ceral, Gopher Bait, Gopher Gitter, Kwik-kill, Stricnina, Mole death, Mouse-nots, Mouse-rid, Mouse-tox, Ro-dex, Strychnos, and Sanaseed. Strychnine poisoning in animals occurs from ingestion of baits designed for use against rodents. The most common domestic animal to be affected is the dog, either through accidental ingestion or through intentional poisoning.

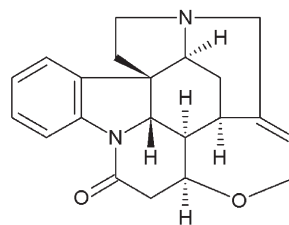


FIGURE 57.1 Structural formula of strychnine.

#### Background

Strychnine was first discovered in Saint-ignatii (*Strychnos ignatii*) beans in 1818 by two French scientists (Joseph-Bienaimé Caventou and Pierre-Joseph Pelletier). *Strychnos ignatii* is a woody climbing shrub found in the Philippines. In general, the beans have the same properties as the species *S. nux-vomica*. Strychnine is also found in other species of *Strychnos* (i.e., *S. colubrine* and *S. tieute*) and is accompanied by another alkaloid brucine. The ripe seeds of *Strychnos* look like flattened discs, which are very hard and covered with satiny hairs. The properties of strychnine are substantially those of the nux-vomica. Strychnine is an extremely toxic alkaloid primarily used to kill rodents, moles, predatory animals, and birds or to trap fur-bearing animals. Pharmaceutically, strychnine is an unjustifiable component of traditional tonics, cathartic pills, and CNS stimulant.

#### Toxicokinetics

Following ingestion, strychnine is readily absorbed from the gastrointestinal (GI) tract, but mainly from the



small intestine. Soon after absorption, strychnine readily distributes to various tissues within 5 min (Reynolds, 1982). Strychnine is readily metabolized in the liver. In fact, the metabolism is rapid enough that approximately two lethal doses can be given over 24 h without cumulative effects. Its half-life has been reported to be approximately 10 h in humans. Elimination of strychnine is also rapid, as its unchanged residue can be detected in the urine within a few minutes of exposure. The elimination constant ( $K_{el} = 0.07\text{h}^{-1}$ ) indicates that 7% of the strychnine in the serum at any one moment would be eliminated in 1 h (Edmunds *et al.*, 1986). Following exposure to a sublethal dose of strychnine, approximately 50% of the dose is eliminated within 6 h (Boyd *et al.*, 1983), 10–20% within 24 h, and almost complete in 48–72 h (Cooper, 1974).

### Mechanism of action

Pharmacologically, because of its bitter taste, strychnine strongly stimulates salivary and gastric secretions. This increases appetite, and as a result, strychnine has been used for a long time to counteract the loss of appetite associated with illnesses.

Strychnine is a potent convulsant. It increases reflex excitability in the spinal cord, which results in a loss of the normal inhibition of spread of motor cell stimulation, so that all muscles contract simultaneously. Strychnine causes excitation of all parts of the CNS. It increases the level of neuronal excitability by interfering with inhibitory influences on the motor neurons. The site of the mechanism of action of strychnine is the postsynaptic membrane. The convulsant action of strychnine is due to interference with the postsynaptic inhibition that is mediated by the amino acid glycine. Glycine is an inhibitory transmitter to motor neurons and interneurons in the spinal cord. Strychnine acts as a selective competitive antagonist to block the inhibitory effects of glycine at the glycine receptors. Studies indicate that strychnine and glycine interact with the same receptor but at different sites. There is also evidence of an increase in brain levels of glutamic acid, an amino acid that acts as a transmitter for excitatory nerve impulses that excite muscle contraction. The result of these effects is that skeletal muscles become hyperexcitable. With a little sound or touch, uncontrollable convulsions and seizures become eminent, followed by suffocation and death. Death occurs due to respiratory failure.

### Toxicity

Strychnine has been studied for acute toxicity in many species, and all species that have been tested have been

TABLE 57.1 Acute toxicity data for strychnine

Species	Route of administration	LD <sub>50</sub> (mg/kg)
Rat	Oral	2.30
Rat	i.p.	2.50
Rat	s.c.	1.20
Rat	i.v.	0.96
Mice	Oral	2.00
Mice	i.p.	0.98
Mice	s.c.	0.474
Mice	i.v.	0.41
Duck	Oral	3.00
Pigeon	Oral	21.00

i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.

From NIOSH (1983–1984).

found to be sensitive. Strychnine is an extremely toxic substance, with an LD<sub>50</sub> of 2.3 mg/kg in rats, 2 mg/kg in mice, 0.6 mg/kg in rabbits, and 0.5 mg/kg in cats and dogs. An approximate lethal dose for a dog is 0.75 mg/kg body weight. Thus, 5-g bait having 0.3% strychnine could be enough to kill a 20-kg dog. Toxicity data of strychnine for various animal species are given in Table 57.1.

Among animals, poisoning occurs with greatest frequency in dogs due to accidental ingestion or malicious intent. The onset of signs can occur within 15–30 min or occasionally 60 min after oral exposure, depending on whether the stomach is empty or full. The clinical signs are associated with CNS effects. Onset of signs includes restlessness, anxiety, muscle twitching, and stiffness of the neck. The poisoned dogs usually show the signs of mydriasis, tonic convulsions, contractions of striated muscles, seizures, opisthotonus, and death. The animal becomes sensitive to touch, sound, noise, or any other sudden change in the environment. A minor stimulation can trigger violent convulsions. Muscular contractions are easily triggered by external stimuli, accompanied by hypothermia, lactic acidosis, rhabdomyolysis and consequent nephrosis, which may result in renal failure (Gupta and Crissman, 2012).

Although strychnine has no direct effects on skeletal muscles, all voluntary muscles contract simultaneously. The increase in muscle tone is caused by the central action of strychnine. The most powerful effects are seen on the muscles of joints. Respiratory muscles (diaphragm, thoracic, and abdominal) contract, respiration ceases, and eventually death ensues due to respiratory failure.

Birds poisoned by strychnine exhibit the signs of ataxia, ruffled feathers, wing droop, salivation, tremors, and convulsions. Death occurs due to respiratory failure. On postmortem, lesions are only observed in the lungs – that is, pinpoint hemorrhages resulting from death due to asphyxia. Rigor mortis occurs soon after death and persists for days. Occasionally, wildlife species are also inadvertently poisoned by strychnine.

There is no evidence of cumulative toxicity from strychnine (Gosselin *et al.*, 1984). From animal studies,

there is no evidence that strychnine has potential for reproductive and developmental toxicity or mutagenic and carcinogenic activity.

### Diagnosis

Diagnosis of strychnine poisoning is based on (1) history of exposure to a strychnine bait or the presence of cracked corn in the digestive tract; (2) clinical signs of tetanic convulsions, seizures, hypersensitivity to external stimuli, and muscle stiffness; and (3) chemical identification of strychnine in the stomach content, blood, urine, or visceral organs (liver and kidney). Strychnine residue can be detected and quantified using gas chromatography (GC)–flame ionization detector or GC–mass spectrometry. It is important to note that strychnine causes elevation of serum enzymes, including glutamic oxaloacetic transaminase, creatine phosphokinase, and lactate dehydrogenase. In addition, lactic acidosis, hyperkalemia, and leukocytosis are the characteristic laboratory findings. In differential diagnosis, tetanus must be ruled out.

### Treatment

There is no specific antidote for strychnine poisoning, so treatment rests with symptomatic and supportive therapies. Seizures need to be controlled as soon as possible with diazepam or phenobarbital. Artificial respiration can be used for apnea. Once the seizures are controlled, detoxification can be performed using gastric lavage with potassium permanganate. Give activated charcoal with a saline cathartic to stop further absorption in the GI tract. Animals should be kept in a quiet environment and should be protected from any secondary sensory input.

### Conclusion

Strychnine is an extremely toxic plant alkaloid that is primarily used as rodenticide. Due to inadvertent use or malicious intent, the poisoning is frequently encountered in dogs. Onset of clinical signs occurs very quickly, and the poisoning is characterized by convulsions, seizures, and hypersensitivity to any external stimulus. Treatment is symptomatic and warranted immediately.

## BROMETHALIN

### Introduction

Chemically, bromethalin is a diphenylamine compound with an appearance of a pale, odorless, and

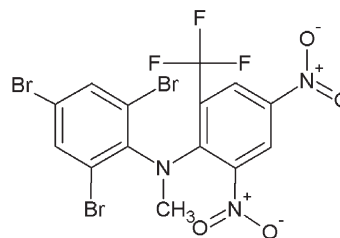


FIGURE 57.2 Structural formula of bromethalin.

solid crystalline powder. It has a chemical formula of C<sub>14</sub>H<sub>17</sub>Br<sub>3</sub>F<sub>3</sub>N<sub>3</sub>O<sub>4</sub>, with a molecular weight of 577.93. Its chemical structure is shown in Figure 57.2. It is commonly used as rodenticide for the control of rats and mice. Bromethalin has many other names and is sold under brand names such as Assault, Trounce, Vengeance, and Wipe Out.

### Background

Bromethalin was developed for use against warfarin-resistant rodents. It is a restricted-use rodenticide used in and around buildings and sewers and inside transportation and cargo vehicles. It is extremely toxic, and only a single dose of bromethalin is needed to cause death in rats and mice. Bromethalin is marketed in a 0.01% formulation (2.84 mg of bromethalin per ounce of bait) and comes in bait pellets, bars, and packs. It is important to note that bromethalin cannot be distinguished from other rodent baits by color or appearance alone. Secondary poisoning usually occurs in dogs and cats that eat a mouse killed by bromethalin.

### Toxicokinetics

Toxicokinetic information on bromethalin is available from laboratory animals such as rats. Bromethalin is rapidly absorbed following oral ingestion. Plasma concentration peaks in approximately 4 h. Metabolism takes place in the liver, where bromethalin undergoes *N*-demethylation, forming desmethyl bromethalin. This metabolite is toxic, as its LD<sub>50</sub> is 7.5 mg/kg body weight. The plasma half-life of bromethalin is approximately 6 days (Dorman *et al.*, 1990c), suggesting slow elimination. Excretion occurs mainly in bile, as it enters into enterohepatic circulation.

### Mechanism of action

Bromethalin is a neurotoxicant and affects the CNS. It uncouples oxidative phosphorylation in the mitochondria,

thereby decreasing ATP synthesis. With a marked decrease in ATP, very little energy is available to maintain  $\text{Na}^+/\text{K}^+$ -ATPase pump. As a result, cells lose their ability to maintain osmotic control,  $\text{Na}^+$  is retained intracellularly, and the cells swell with water. This leads to fluid accumulation within myelin sheaths and vacuolation of the nervous system, resulting in nerve conduction impairment. Damage to neuronal axons and increased intracranial pressure occur, followed by convulsions, paralysis, and death (Dorman *et al.*, 1992).

## Toxicity

Bromethalin is a single-dose rodenticide. It is classified as highly toxic if swallowed, inhaled, or absorbed through skin. The acute oral  $\text{LD}_{50}$  of bromethalin is 2.38–5.6 mg/kg in bait for dogs and 0.4–0.71 mg/kg in bait for cats (Dorman *et al.*, 1990c). Its  $\text{LD}_{50}$  is 2 mg/kg in mice, 5 mg/kg in rats, 13 mg/kg in rabbits, and 0.25 mg/kg in pigs.

In field cases, most often poisoning occurs in pets, including dogs and cats. Signs of bromethalin appear within 10 h to several days after exposure and may last up to 12 days. In most cases, the poisoning is acute in nature, characterized by cerebral edema and paralysis of the hind limbs. In general, clinical signs in pets include severe muscle tremors, hyperexcitability, hyperesthesia (hypersensitivity to touch), and seizures. Symptoms with mild exposure to bromethalin occur with slow progress in several days, and they include loss of ability to bark, loss of appetite, vomiting, depression, lethargy, tremors, paralysis, lateral recumbency, coma, and death. Following exposure to a large dose of bromethalin, animals can show signs of muscle tremors and seizures, hyperexcitability, ataxia and paddling, hyperthermia, potential loss of vocalization, loss of tactile sensation, and forelimb extensor rigidity (Schiff–Sherrington posture), and death occurs within 2–4 days. Death can occur with a low or high dose, and is usually caused by respiratory paralysis. Poisoned dogs show the signs of tremors, ataxia, depression, tachypnea, hyper-reflexia of the hind limbs, loss of vocalization, recumbency, anorexia, vomiting, and death (Dorman *et al.*, 1990a). Poisoned cats exhibit the signs of ataxia, seizures, vocalization, rigidity, decreased proprioception, abdominal distension, recumbency, depression, and death (Dorman *et al.*, 1990b, 1992). Other signs of poisoning include generalized seizures, head pressing, hyperesthesia, coma, hyperexcitability, ataxia, extensor rigidity, nystagmus, hyperthermia, cyanosis, miosis, and drooling (Moorman, 2003). Overall, cats are much more sensitive than dogs to bromethalin.

Histopathological changes have been described in dogs receiving a single oral dose of bromethalin

(6.25 mg/kg). Histologic lesions included diffuse white matter spongiosis, mild microgliosis, optic nerve vacuolization, mild thickening of Bowman's capsule, and occasional splenic megakaryocytes. Ultramicroscopic examination of mid-brain stem revealed occasional swollen axons, intramyelinic vacuolization, and myelin splitting at the intraperiod line (Dorman *et al.*, 1990c). Dorman *et al.* (1992) also reported histopathological changes in cats induced by bromethalin. In brief, ultrastructural changes include separation of myelin lamellae at the interperiod lines with the formation of intramyelinic vacuoles (intramyelinic edema), rupture and coalescence of intramyelinic vacuoles into larger extracellular spaces (spongy change), and pronounced cytosolic edema of astrocytes and oligodendroglial cells. Histopathology of the brain and spinal cord of rodents receiving multiple low or sublethal doses of bromethalin revealed a spongy degeneration of the white matter that was shown upon ultramicroscopic examination to be intramyelinic edema (Van Lier and Cherry, 1988).

According to the World Health Organization and the U.S. Environmental Protection Agency, bromethalin is considered carcinogenic.

## Diagnosis

Diagnosis of bromethalin poisoning is based on history of exposure to bromethalin bait, clinical signs, and identification of bromethalin in bait, GI content, brain, and visceral organs. Residue of bromethalin or its major metabolite (desmethyl bromethalin) can be quantified using GC coupled with electron capture detector (Dorman *et al.*, 1990c) or high-performance liquid chromatography (HPLC) coupled with ultraviolet detector or with negative-ion atmospheric pressure chemical ionization–mass spectrometric detector (Mesmer and Flurer, 2001). The highest concentrations of bromethalin are found in fat, liver, kidney, and brain. Differential diagnosis should rule out lead, ethylene glycol, organophosphates, strychnine, metaldehyde, zinc phosphide, and tremorgenic mycotoxins.

## Treatment

There is no specific antidote for bromethalin poisoning. Symptoms can be treated with corticosteroids, but clinical studies indicate that symptoms return as soon as the corticosteroids are discontinued. Emesis should be induced using apomorphine or 3% hydrogen peroxide if the animal is not exhibiting signs of convulsions and seizures. Alternatively, perform gastric lavage and give activated charcoal with saline cathartic. Activated charcoal needs to be repeated if the dog or cat is exposed to a large dose of bromethalin. The animal needs to be

monitored and treated for cerebral edema. Intravenous fluids can be administered with great caution so as not to worsen cerebral edema. Diazepam or barbiturate can be given to control seizures.

## Conclusion

Bromethalin is a commonly used rodenticide that is encountered in poisoning in dogs and cats. Bromethalin exerts toxicity by uncoupling oxidative phosphorylation in mitochondria, thereby decreasing ATP synthesis. The CNS is the target organ, and toxicity is characterized by cerebral edema, convulsions, and paralysis. There is no specific antidote, so treatment is symptomatic and supportive.

## CHOLECALCIFEROL

### Introduction

Cholecalciferol is a form of vitamin D, also called vitamin D<sub>3</sub>, that is commonly used as rodenticide. Vitamin D<sub>3</sub> is a secosteroid and structurally similar to other steroids, such as cholesterol, testosterone, and cortisol. It has chemical formula C<sub>27</sub>H<sub>44</sub>O, with a molecular weight of 384.64. Its structural formula is shown in Figure 57.3. Cholecalciferol has other names and is marketed as a rodenticide under the brand names Quintox, True Grit Rampage, and Ortho Rat-B-Gone; it is also marketed as a feed additive under the name Viactive.

### Background

Cholecalciferol is a rodenticide that is used in and around buildings and inside transport vehicles. Vitamin D<sub>3</sub>, as such, does not have significant biological activity,

but in two steps, it is metabolized in the body to make an active form. In liver, cholecalciferol is hydroxylated to 25-hydroxycholecalciferol (calcifediol) by the enzyme 25-hydroxylase. In kidney, 25-hydroxycholecalciferol serves as a substrate for 1- $\alpha$ -hydroxylase, forming 1,25-dihydroxycholecalciferol (calcitriol), which is the biologically active form. Cholecalciferol is formulated in the granular form (0.075% bait) and is very effective against Norway rats, roof rats, and house mice.

### Toxicokinetics

Very little information is available on the toxicokinetics of cholecalciferol. Cholecalciferol is transported in blood bound to carrier proteins. The major carrier protein is vitamin D binding protein. The half-life of 25-hydroxycholecalciferol is several weeks, whereas that of 1,25-dihydroxycholecalciferol is just a few hours.

### Mechanism of action

Ingestion of cholecalciferol-containing bait is known to cause a marked increase in calcium level in blood and tissues. Cholecalciferol not only increases the absorption of calcium but also mobilizes calcium and phosphorus from bones to the circulation. High calcium causes heart problems and bleeding secondary to mineralization of the vessels, kidneys, stomach wall, and lungs. Mineralization of the kidney leads to renal failure and death. Cholecalciferol and its metabolites exert their effects by binding to vitamin D receptors in tissues. 1,25-Dihydrocholecalciferol is the most metabolically active form, with 500 times greater binding to the vitamin D receptors than that of 25-hydroxycholecalciferol and 1000 times greater binding than that of cholecalciferol.

### Toxicity

Cholecalciferol is of low toxicity to mammalian species, as it is classified as a class III toxic chemical. The oral LD<sub>50</sub> of cholecalciferol in rats is 43.6 mg/kg and in mice is 42.5 mg/kg. The dermal LD<sub>50</sub> in rabbits is 2000 mg/kg. Studies suggest that cholecalciferol is of low toxicity to birds (oral LD<sub>50</sub> is >2000 mg/kg in mallard ducks and dietary LC<sub>50</sub> is 4000 ppm in mallard ducks and 2000 ppm in bobwhite quail).

Pets such as dogs are poisoned by ingesting rodenticide bait, whereas farm animals are affected by overdose of additive vitamin D<sub>3</sub> in the feed. Signs and symptoms of poisoning are similar to those of hypercalcemia, such as anorexia, fatigue, headache, itching, weakness,

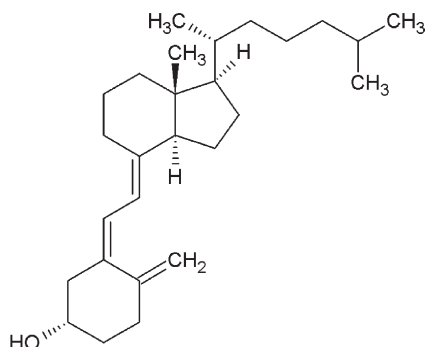


FIGURE 57.3 Structural formula of cholecalciferol.



nausea, vomiting, and diarrhea. In acute cases, cholecalciferol causes severe polyneuropathy. Dogs poisoned with cholecalciferol-containing rodenticide bait usually show signs of depression, anorexia, vomiting, bloody diarrhea, cardiac irregularities, hypertension, seizures, and death. In dogs, signs of poisoning may occur with as little as a 2 mg/kg dose, and death may occur with a 10 mg/kg dose of cholecalciferol.

## Treatment

Decontamination, including induction of emesis, gastric lavage, and administration of activated charcoal, is beneficial. Administration of intravenous fluids helps reduce serum calcium levels by increasing urine production and calcium excretion. Biphosphonate pamidronate disodium is used to decrease serum calcium levels. Seizures can be controlled by diazepam or barbiturates. Treatment needs to be continued for 2 or 3 weeks because the elimination half-life of cholecalciferol is more than 2 weeks. Poisoned animals should receive feed devoid of vitamin D<sub>3</sub>.

## Conclusion

Cholecalciferol is a single- or multiple-dose rodenticide that has low toxicity to mammalian and avian species. It produces toxicity by marked increases in calcium and mineralization of tissues. Once animals develop severe signs, they usually die.

# RED SQUILL

## Introduction

Red squill, which is also known as sea onion, is obtained in the powder form from the plant *Urginea maritima*. The plant is native to the Mediterranean region. It resembles an onion, and its bulb extracts and dried powders have been used for the control of rodents since the 13th century. Although red squill has many alkaloids, scilliroside is the most toxic and provides rodenticidal activity. It has the chemical formula C<sub>32</sub>H<sub>44</sub>O<sub>12</sub> and a molecular weight of 620.7. Its structural formula is shown in Figure 57.4. The compound is sparingly soluble in water and thermostable. Scilliroside is formulated in the powder form (Dethdiet) and liquid extract (Rodine), and it has many other names, including Sea squill, Scilla maritima, Silmurin, Silmine, Sea onion, and Squill. It was demonstrated long ago by Winton (1927b) that only red squill, and not white squill, has rodenticide activity. The red squill is mixed in baits

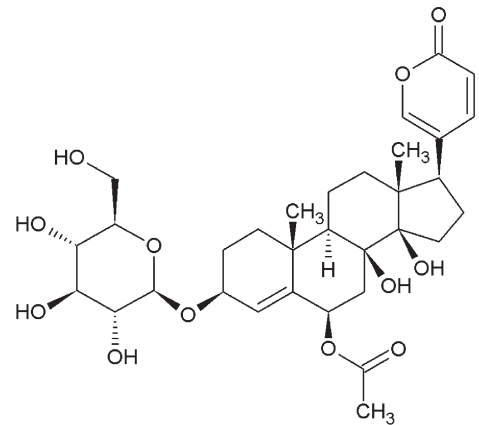


FIGURE 57.4 Structural formula of scilliroside.

and applied at a 10% concentration and mixed with meat, fish, and cereals (Thomson, 1991–1992).

## Background

Red squill bulbs were an ancient source of rodenticide products replaced later by warfarin and other modern anticoagulant rodenticides. Because rats have developed resistance to such products, there is renewed interest in the red squill. Studies suggest that the white squill drug is used as a diuretic, stimulant, and expectorant, whereas the red squill is used mostly as a rat poison. Many glycosides and aglycones have been isolated from the red squill bulb. Krenn *et al.* (1994) and Pascual-Villalobos (2002) isolated five different bufadienolides as pure substances (proscillaridin A, scillaren A, scilliroside, gam-mabufotalin, and scillirosidin). Scilliroside is the most toxic bufadienolide glycoside (Verbiscar *et al.*, 1986a,b).

Scilliroside has an emetic property. Thus, if rodents ingest a product containing scilliroside, because they are incapable of vomiting, they develop glycoside intoxication and pulmonary edema. However, the compound has rarely been associated with toxicity in humans because humans are capable of vomiting and exhibit poor gastrointestinal absorption.

## Toxicokinetics

Scilliroside is inefficiently absorbed from the GI tract. Its metabolism is not well studied, although the unmetabolized scilliroside is rapidly excreted in the urine.

## Mechanism of action

Red squill bulbs contain many glycosides, but scilliroside is the major component that has a rodenticidal

property. The compound is cardiotoxic, similar to digitalis; that is, injection of scilliroside induces cardiac impulse condition and arrhythmias. In very early experiments, red squill preparations administered to rats induced convulsions and paralysis (Winton, 1927a). Female rats succumb to red squill at half the dose that is required to kill males.

## Toxicity

The red squill plant (mainly the bulb) contains scilliroside, a highly toxic bufadienolide glycoside. It adversely affects cardiovascular and central nervous systems, causing convulsions and death. The oral LD<sub>50</sub> of scilliroside is 0.7mg/kg in male rats and 0.43mg/kg in female rats, and it is 0.35mg/kg in mice. Studies found that pigs and cats survived doses of 16 mg/kg and fowls survived 400mg/kg (Worthing, 1983). It is classified as class I – that is, a highly toxic chemical.

Red squill contains several compounds that have emetic properties. Due to poor gastrointestinal absorption and decreased potency, red squill has seldom been associated with toxicity in humans, dogs, cats, and pigeons. However, rats and mice are unable to vomit, and they die within a few hours after ingesting a lethal dose of scilliroside.

In toxic doses, red squill produces inflammation of the gastrointestinal and genitourinary tracts, manifested by nausea, vomiting, abdominal pain, and purging. Other signs of poisoning include convulsions, hypothermia, enfeebled circulation, blurred vision, and sometimes death. Convulsions are seen in humans, and they have also been observed in rats. Higher doses of red squill can cause serious heart rhythm alterations resulting in death. Farm animals require large quantities for intoxication. There are field cases in which dogs, cats, and pigs have been poisoned. Signs of poisoning include vomiting, ataxia, and hyperesthesia, followed by paralysis, depression, or convulsions. Cardiac arrest occurs due to bradycardia and cardiac arrhythmias. Generally, animals exposed to a sublethal dose can recover in less than 48 h.

## Treatment

Treatment is based on symptomatic and supportive therapies. In case a significant amount of red squill is retained in the stomach, decontamination (gastric lavage with saline cathartic) is rewarding. The patient must be monitored for cardiac arrhythmias and conduction disturbances. Atropine sulfate given subcutaneously at 6- to 8-h intervals may prevent cardiac arrest. Phenytoin at 35mg/kg, TID, should be given to dogs to suppress arrhythmias.

## Conclusions

Red squill is a botanical rodenticide and exerts toxicity due to cardiac effects. Treatment is symptomatic and supportive.

# FLUOROACETATE

## Introduction

Sodium fluoroacetate (compound 1080) is an extremely toxic white powder that has been used as a rodenticide throughout the world. The compound has the chemical formula C<sub>2</sub>H<sub>2</sub>FO<sub>2</sub>.Na, with a molecular weight of 100.02. Its structural formula is shown in Figure 57.5. It is used to control rats, mice, squirrels, prairie dogs, foxes, wolves, coyotes, and rabbits. The compound is also used to control brush-tail possums, deer, wild pigs, wallabies, and rooks. It is very toxic to birds, domestic animals, and carnivores. Secondary poisoning is very common in birds and carnivores from eating poisoned carcasses.

Sodium fluoroacetate has many other names and is sold under trade names such as Nissol, 1080 gel, 1080 paste, 1080 solution, Tenate, and Tenate 1080. The commercial products are provided with a black dye called nigrosine (0.5%).

## Background

Sodium fluoroacetate was discovered by German military chemists during World War II. However, it was not until later that American chemists discovered its use as a rodenticide. The name “1080” refers to the catalog number of the poison, which became its brand name. The compound is an extremely toxic substance, which is commonly used as a rodenticide. Inhalation of dust or swallowing can also be fatal. The compound can also be absorbed through cuts or abrasions in the skin and lead to poisoning.

It is important to note that sodium fluoroacetate is also formed naturally in approximately 40 plants that are native to Australia, Brazil, and Africa after fluoride uptake from soil, water, or air. Examples of plants that contain sodium fluoroacetate are *Dichapentalum cymosum*, *D. toxicarum*, *Chaillietia toxicaria*, *Gastrolobium grandiflorum* (“poison peas”), *Oxylobium parviflorum*, and

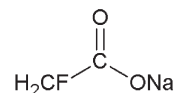


FIGURE 57.5 Structural formula of sodium fluoroacetate.

*Acacia georginae*. Consumption of these plants has resulted in many serious cases of livestock poisoning and high stock losses (Oelrichs and McEwan, 1962). Poisoning has also been documented in field-workers exposed to fluoroacetate (Suh *et al.*, 1970).

## Toxicokinetics

Sodium fluoroacetate (1080) is rapidly absorbed from the GI tract. Dust formulations are easily absorbed by inhalation, which is not usually the route for poisoning cases. 1080 is not readily absorbed through intact skin, but it can be absorbed in the case of cuts, abrasions, or dermatitis (Brockman *et al.*, 1955). It is reported that sublethal doses of compound 1080 are completely metabolized and excreted in 4 days.

The bioavailability of sodium fluoroacetate appears to be similar for oral, injected, and inhaled doses. Dermal absorption is lower because a subcutaneous LD<sub>50</sub> is 10- to 15-fold higher than the oral dose. Distribution studies suggest that the plasma levels of sodium fluoroacetate are twice those of tissues. Sheep receiving sodium fluoroacetate (0.1 mg/kg) contained the residue in plasma, kidney, heart, muscle, spleen, and liver as 0.098, 0.057, 0.052, 0.042, 0.026, and 0.021 (Eason *et al.*, 1993). The plasma  $t_{1/2}$  is 3.6–6.9 h in goats, 6.6–13.3 h in sheep, 1.1 h in rabbits, and 1.6–1.7 h in mice (Eason *et al.*, 1993; Gooneratne *et al.*, 1995). Metabolism of fluoroacetate (i.e., defluorination) takes place in the liver. Fluoroacetate and fluorocitrate salts are excreted mainly in the urine.

## Mechanism of action

In the body, fluoroacetate (1080) is converted to fluorocitrate, which is a potent inhibitor of the enzyme aconitase in the tricarboxylic acid cycle (Krebs cycle) (Elliot and Kalnitsky, 1996). As a result, the elevated levels of citrate in blood become observable 30 min and maximum levels at 4 h after administration. It has been shown that citrate levels are directly influenced by thyroid hormone (i.e., free T<sub>3</sub>; Maruo *et al.*, 1992). Accumulation of citrate causes toxicity due to reduction of ATP levels by inhibiting energy production in most cells of the body, leading to a slow and painful death as the body “suffocates from within.” Compound 1080 causes damage to tissue of high energy needs, such as brain, heart, lungs, and fetus.

Accumulated levels of citrate cause chelation of divalent metal ions, especially Ca<sup>2+</sup>. Depletion of these ions in the CNS may be responsible for seizures in certain species (Hornfeldt and Larson, 1990).

Fluoroacetate/fluorocitrate also affects activities of other enzymes, including mitochondrial citrate carriers, pyruvate dehydrogenase kinase (Taylor *et al.*, 1977),

succinate dehydrogenase (Mehlman, 1967), glutamine synthetase, phosphofructokinase (Godoy and del Carmen Villarruel, 1974), and ATP-citrate lyase (Rokita and Walsh, 1983).

## Toxicity

Most species are sensitive to fluoroacetate (1080); however, rodents and dogs are the most sensitive species. The oral LD<sub>50</sub> of 1080 is 0.1–0.22 mg/kg in rats, 0.1 mg/kg in mice, 0.34 mg/kg in rabbits, and 0.3 mg/kg in guinea pigs. The oral LD<sub>50</sub>s of this compound in the house sparrow, red-winged blackbird, starling, and golden eagle are 3.0, 4.22, 2.37, and 1.25–5 mg/kg, respectively. Measured LD<sub>50</sub> of this rodenticide in mammalian wildlife is 0.22–0.44 mg/kg in mule deer, 1.41 mg/kg in male ferrets, and 0.5–1.0 mg/kg in bears.

In general, fluoroacetate is very toxic to mammalian, bird, and wildlife species, whereas it is of low toxicity to fish. Toxicity of 1080 is different according to route of exposure (i.e., ingestion or inhalation), and symptoms vary widely among species. Species have been categorized into four groups according to symptomatology:

1. Rabbit, goat, horse, sheep, and spider monkey: CNS effects are not observed, and death is due to cardiac effects with ventricular fibrillation.
2. Cat, pig, rhesus monkey, and human: Heart and CNS are affected, and death usually results from respiratory failure during convulsions but is occasionally due to ventricular fibrillation.
3. Dog and guinea pig: Epileptiform convulsions predominate, with death being due to cessation of respiratory activity following running movements such as those of strychnine poisoning.
4. Rat and hamster: Respiratory depression and delayed bradycardia are the main features.

In general, 1080 produces convulsions, involuntary urination, vomiting, and ventricular fibrillation. The onset of symptoms of poisoning is usually between 30 min and 4 h after exposure. The common symptoms are vomiting, involuntary hyperextension of the limbs, convulsions, and, finally, cardiac and respiratory failure. Dogs usually show CNS signs such as convulsions and uncontrollable running, whereas sheep and cattle show predominantly cardiac signs. Dogs appear to be highly sensitive to fluoroacetate, and mass poisonings of dogs eating contaminated poultry have been documented (Egyed, 1979).

The main target organs affected are the central nervous, cardiovascular, and respiratory systems. This causes metabolic derangement that includes alteration in transaminase, calcium, and glucose levels apart from acidosis and renal failure.

Clinic effects are associated with neurological and cardiac systems. CNS effects include tremulousness, hallucinations, convulsions, and respiratory depression. Cardiac effects include arrhythmias, ventricular fibrillation, and cardiac arrest. If the patient survives the first 24h after ingestion of sodium fluoroacetate, recovery is favorable. Acute exposure often results in complete recovery or death. Of course, in some cases, exposure results in cardiac damage.

Severity of signs is dose related. The oral route is the most important in cases of poisoning of 1080. Dust formulations are easily absorbed by inhalation, which is not usually the route for poisoning cases. Compound 1080 is not readily absorbed through intact skin, but it can be absorbed in the case of cuts and dermatitis.

The fluorocitric acid is itself highly toxic, and therefore sodium fluoroacetate can cause secondary poisoning – that is, poisoning in an organism that has consumed a part of an organism already poisoned.

The development of tolerance to increasing doses of fluoroacetate has been reported in rats and mice, whereby a dose of 0.5mg/kg protects rats against a dose of 5mg/kg for a period of 48h (Chenoweth, 1949). The mechanism of fluoroacetate resistance in certain species is not well understood, but the rate of defluorination does not appear to play a significant role (Mead *et al.*, 1985).

Studies suggest that sodium fluoroacetate has no carcinogenic, mutagenic, or teratogenic potential.

### Diagnosis

Diagnosis is based on evidence of exposure, clinical signs, necropsy findings, and chemical confirmation. Samples for chemical confirmation should include suspected bait, vomitus, stomach content, liver, and kidney. Testing for 1080 should be performed on the vomited stomach contents. In the case of ruminants, rumen content needs to be analyzed for fluoroacetate. Significant elevation of citric acid levels in blood and kidney is a reliable biochemical marker of fluoroacetate or fluorocitrate poisoning (Basakowski and Levin, 1986). Hyperglycemia, hypocalcemia, and hypokalemia are characteristic laboratory findings. Other metabolic/biochemical changes include metabolic acidosis resulting from a buildup of citric acid, lactic acid, and ammonium in blood and organs. Metabolic acidosis is also associated with elevated serum creatinine and transaminase levels.

Differential diagnosis should include lead, strychnine, chlorinated hydrocarbons, and plant alkaloids.

### Treatment

There is no specific antidote for 1080 poisoning. Use of glyceryl monoacetate has shown some positive results

because it provides acetate ions to allow continuation of the cellular respiration process, which is interrupted by compound 1080. Other symptomatic and supportive measures following decontamination procedures (to prevent further absorption by activated charcoal) include use of anticonvulsants, muscle relaxants, and mechanical ventilation. Induction of emesis is contraindicated because of potential arrhythmias and convulsions. Special attention should be paid to stabilize cardiac and CNS functions.

Acetate and ethanol have been found to be potentially effective in mice, guinea pigs, and rabbits but not in dogs. A combination of calcium gluconate and sodium succinate has proven effective in mice (Omara and Sisodia, 1990). Calcium chloride has antidotal effects in cats (Roy *et al.*, 1980).

### Conclusion

Fluoroacetate converts to fluorocitrate, which is an extremely toxic metabolite. Symptoms vary markedly between species. For example, carnivores exhibit more signs related to the CNS, herbivores exhibit signs related to cardiac effects, and omnivores show signs of both CNS and cardiac effects. Treatment relies on symptomatic and supportive measures.

## ALPHA-NAPHTHYL THIOUREA

### Introduction

Alpha-naphthyl thiourea (ANTU) is a colorless, odorless, crystalline powder that is exclusively used as rodenticide. The technical product is gray powder. Its chemical formula is  $C_{11}H_{10}N_2S$ , and it has a molecular weight of 220.28. Its structural formula is shown in Figure 57.6.

ANTU has several other names, including alpha-naphthyl thiocarbamide, 1-naphthyl-thiourea, *N*(1-naphthyl)-2 thiourea, Alrato, Anturat, Bantu, Dirax, Krysids, Rat-tu, and Rattrack.

### Toxicokinetics and mechanism of action

Following ingestion, ANTU is rapidly absorbed from the GI tract. The exact mechanism of action of ANTU is not known. ANTU stimulates the sympathetic nervous system and causes a major increase in the permeability of the lung capillaries; consequently, extensive pleural effusion and pulmonary edema develop. This results in respiratory failure.



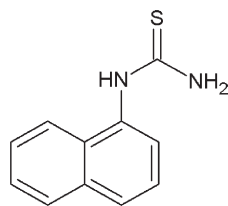


FIGURE 57.6 Structural formula of alpha-naphthyl thiourea.

## Toxicity

All animals are susceptible to ANTU, but lethal doses differ widely. Lethal doses of ANTU (in mg/kg) in various species are as follows: rats (3), dogs (10), pigs (25), horses (30), cows (50), cats (75), and fowl (2500).

Clinical signs of poisoning due to ANTU include vomiting, abdominal pain, dyspnea, shortness of breath, seizures, bluish discoloration, coarse pulmonary rales, pulmonary edema, and liver damage. In a time course study, ANTU at a dose rate of 5mg/kg induced lung edema in adult albino rats (Vivet *et al.*, 1983). After 6h, pulmonary extravascular water increased by 50% in ANTU-treated rats, and the volume of the pulmonary effusion reached  $3.4 \pm 0.1$  mL. The most characteristic feature is the absence of hypoxemia in ANTU-intoxicated rats. The absence of hypoxemia is common with normobaric oxygen. ANTU can produce hyperglycemia of three times normal in 3h.

Chronic sublethal exposure to ANTU may cause enlarged thyroid gland (goiter) and interfere with normal thyroid function. Repeated sublethal doses in rats lead to the development of tolerance so that resistance to several lethal doses develops (Peoples, 1970).

## Diagnosis

Diagnosis of ANTU poisoning is based on evidence of rodenticide exposure, clinical signs, lung and liver damage, and chemical confirmation. ANTU can be quantified using HPLC.

## Treatment

There is no specific antidote. Therefore, treatment relies on symptomatic and supportive measures. Induce vomiting if the patient is not showing convulsions and seizures.

The decontamination procedure includes administration of activated charcoal.

# ZINC PHOSPHIDE

## Introduction

Zinc phosphide is an inorganic compound with the appearance of gray crystalline powder and a decaying

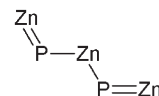


FIGURE 57.7 Structural formula of zinc phosphide.

fish or garlic odor. It has the chemical formula  $Zn_3P_2$ , with a molecular weight of 258.1. Its structural formula is shown in Figure 57.7. Zinc phosphide was first synthesized in 1740 and was first used as a rodenticide in 1911–1912 in Italy. It was not used in the United States until 1939. In the 1970s, there was a renewed interest in zinc phosphide for rodent control in agriculture. Currently, zinc phosphide is used worldwide for the control of a range of animals, including mice, rats, voles, ground squirrels, prairie dogs, moles, muskrats, rabbits, and gophers. Pelleted zinc phosphide is sold extensively under brand names such as Arrex, Blue-ox, Gopharid, Kil-rat, Mous-con, Phosvin, Pollux, Ratol, Ridall, Rodenticide AG, Zinc-tox, and ZP. It is also used on crop/noncrop areas, including lawns, golf courses, and around wetlands.

## Background

The history of the use of zinc phosphide as a rodenticide dates back approximately a century. In the early years, technical zinc phosphide and highly concentrated paste were sold in shops and used by consumers to make their own baits for rodents. This led to use of the technical material for suicide and to accidental deaths in small children and pets.

Zinc phosphide is recognized as the slowest acting of the commonly used rodenticides. It can cause toxicity and death in rodents after one feeding if adequate dose is consumed. However, bait must be continuously available to be effective. In fact, zinc phosphide is recommended as the rodenticide of choice because it is fairly specific for rodents and secondary poisoning is rare, except in dogs and cats.

Rodenticide-grade zinc phosphide usually comes as a black powder containing 75% zinc phosphide and 25% antimony potassium tartrate, an emetic to cause vomiting if the material is accidentally ingested by humans or domestic animals.

## Toxicokinetics

Zinc phosphide can be absorbed into the body by oral ingestion, inhalation, and through damaged skin. Following oral ingestion, zinc phosphide reacts in the stomach and intestine with water and hydrochloric acid to liberate phosphine gas. Metabolism of zinc phosphide

can occur via oxidation of the phosphorus to various phosphorus oxyacids or via reduction of the phosphorus to phosphine gas. Zinc phosphide excretes in the urine either as a hypophosphite or as dissolved phosphine. The presence of strong reducing substance in the urine is a common feature of poisoning with zinc phosphide. Other metabolites include phosphoric acid and phosphate. Phosphine gas is also exhaled from the lungs.

### Mechanism of action

The overall toxicity of zinc phosphide is due to both zinc and phosphine gas, but it is primarily from phosphine. Phosphine can enter the bloodstream and adversely affect the lungs, liver, kidneys, heart, and CNS. Phosphine causes CNS depression, irritation of the lungs, damage to the blood vessels and erythrocyte membranes, and eventually cardiovascular collapse and irritation of the alimentary tract. Zinc phosphide also causes damage to the liver and kidney.

### Toxicity

Zinc phosphide is an extremely toxic compound. The oral LD<sub>50</sub> in rats is 41 mg/kg body weight. In sheep, the LD<sub>50</sub> ranges from 60 to 70 mg/kg. It is also very toxic to cows, sheep, goats, pigs, rabbits, and other species. Following oral ingestion, both zinc phosphide and phosphine are absorbed from the GI tract, although the majority of acute effects are caused by phosphine. Zinc phosphide causes damage to the liver and kidney. Phosphine causes CNS depression, irritation of the lungs, and damage to the liver, kidney, heart, and CNS. Death occurs as a result of cardiac arrest or, more commonly, pulmonary edema. In addition, there are reports of cardiac arrest accompanied by kidney damage. Following a large dose, death usually occurs within 1 h, whereas with smaller doses, death can occur between 4 and 72 h. Symptoms usually appear 20–25 min after exposure. Animals are prostrated with deep slow respiration, finally terminating in convulsions. Following repeated exposure, cumulative effects occur in the liver, kidney, and lungs.

Zinc phosphide is highly toxic to wild animals and birds and to freshwater fish. The most sensitive bird species that have been evaluated are geese (LD<sub>50</sub> of 7.5 mg/kg). Pheasants, mourning doves, quails, mallard ducks, and horned larks are very sensitive to this rodenticide. The fish species that have been found sensitive to zinc phosphide include bluegill sunfish, rainbow trout, and carp.

### Diagnosis

Diagnosis of zinc phosphide in animals is based on detection of zinc phosphide, phosphine, and zinc in

body tissues and fluids. At necropsy, stomach content smells like acetylene. A zinc phosphide level of 50 ppm or higher in stomach content is considered significant and is indicative of zinc phosphide poisoning.

### Treatment

There is no specific antidote, and treatment is mainly symptomatic. Vomiting should be induced as soon as possible after ingestion, followed by the administration of activated charcoal. Sodium bicarbonate can be given orally to stop liberation of phosphine gas. Calcium gluconate and sodium lactate can be given intravenously to combat acidosis.

### Conclusion

Zinc phosphide is a slow-acting but highly toxic rodenticide. At acidic pH in stomach, zinc phosphide generates phosphine gas, which is responsible for the majority of toxic effects. There is no specific antidote, and treatment is symptomatic.

## THALLIUM

### Introduction

Thallium (Tl) is a bluish-white heavy metal that occurs naturally in the earth's crust. The word thallium derives from the Greek word *thallos*, which means a young twig or shoot. Tl enters the environment from natural and anthropogenic sources. Natural sources of Tl are less bioavailable and therefore of less toxicological concern than anthropogenic sources. The largest anthropogenic sources of Tl are related to coal combustion and heavy metal (primarily zinc and cadmium) smelting and refining. Tl salts were introduced as pesticides in Germany in 1920. The sulfate salt is most common and has been widely used as a rodenticide and ant killer. In the past, Tl was also used for medicinal purpose against dysentery with violent or persistent diarrhea, syphilis, gonorrhea, gout, mycosis of the scalp, and as an inhibitor of sweat secretion in tuberculosis patients. Approximately 50 years ago, Tl was frequently referred to as the poison of choice because Tl is as toxic as arsenic or lead. Tl is banned in many countries, but it is still widely available in developing countries as a rodenticide. The possibility also exists for Tl to be used as a chemical warfare agent (Thompson, 2009). In general, Tl poisoning cases are on the decline, but diagnostic labs still receive suspected baits and tissues from poisoned animals, especially dogs, on a regular basis.

## Background

Thallium is a toxic heavy metal that was discovered by Sir William Crookes in 1961 by burning the dust from a sulfuric acid industrial plant. It forms two kinds of compounds: monovalent thallo- and trivalent thalli- compounds. The monovalent Tl resembles potassium, and trivalent Tl resembles aluminum. Tl tends to form stable complexes with soft ligand donors, such as sulfur-containing compounds. Inorganic Tl (I) compounds are more stable than Tl (III) analogs in aqueous solution at neutral pH. In contrast, organothallium compounds are stable only in the trivalent form. Tl is particularly toxic in its Tl (I) compounds, such as sulfate ( $\text{Tl}_2\text{SO}_4$ ), acetate ( $\text{CH}_3\text{COOTl}$ ), and carbonate ( $\text{Tl}_2\text{CO}_3$ ). The sulfide ( $\text{Tl}_2\text{S}$ ) and iodide (Tl I) are both poorly soluble and therefore much less toxic. Today, Tl is recognized as one of the most toxic heavy metals, with an  $\text{LD}_{50}$  of 30 mg/kg in rats and 8–12 mg/kg in humans.

## Toxicokinetics

The water-soluble Tl compounds are rapidly absorbed following oral, inhalation, and dermal exposure. After absorption, Tl compounds are widely distributed to the body tissues, including brain, heart, kidney, skeletal muscle, and testes. Both monovalent and divalent Tl appear to distribute in tissues in a similar manner. In blood, Tl is found slightly more within erythrocytes than in plasma. Following an acute exposure, the maximal concentration of Tl is found in the kidneys, preferentially in the medulla. Studies conducted in rats show lower concentrations of Tl in renal tissue of young compared to adult.  $\text{Tl}^+$  and  $\text{K}^+$  are monovalent cations with similar ionic radii, but  $\text{Tl}^+$  accumulates intracellularly more than  $\text{K}^+$  because of its greater affinity for certain enzymes and protein. Because of its large volume of distribution and low free plasma concentration, renal excretion of Tl is slow, and its residue can be detected in the tissues for months. Organic Tl compounds, such as thallous malonate, show a higher elimination rate constant but are similar in toxicity and distribution pattern compared to the inorganic Tl compounds, such as thallous sulfate (Aoyama, 1989). The elimination half-life is between 8 and 30 days. Tl is excreted in urine, bile, feces, saliva, tears, and milk. In mammals, Tl excretion via the GI tract is twice that of the kidneys because of its involvement in enterohepatic circulation. Tl is known to pass in the milk of poisoned female rats, mice, guinea pigs, and humans. A significant fraction of free plasma Tl also crosses the placental barrier. A small amount of Tl can also be found in hair. Following chronic exposure, Tl can be deposited in the bones, which may account for its cumulative toxicity.

## Mechanism of action

Tl produces toxicity in mammals through multiple mechanisms.  $\text{Tl}^+$  and  $\text{K}^+$  have common cellular targets and receptor sites associated with biological activity and toxicity.  $\text{Tl}^+$  replaces  $\text{K}^+$  in the intracellular environment and shows a 10-fold greater affinity over  $\text{K}^+$  for  $\text{Na}^+/\text{K}^+$ -ATPase. Tl at higher concentrations competitively inhibits  $\text{Na}^+/\text{K}^+$ -ATPase activity. Mitochondria have an abundance of  $\text{Na}^+/\text{K}^+$ -ATPase and are particularly susceptible to the effects of Tl. Tl inhibits the influx and efflux of  $\text{K}^+$  in mitochondria, without causing any alterations in movement of  $\text{Na}^+$ . In addition, Tl inactivates sulfhydryl groups, including those affecting the permeability of the outer mitochondrial membrane. As a result of mitochondrial dysfunction, a variety of morphological changes, including mitochondrial swelling and vacuolization, occur in mitochondria in kidney, liver, brain, pancreas, and other tissues of mammals. Tl compromises mitochondrial energy production by inhibiting pyruvate dehydrogenase complex and succinate dehydrogenase by uncoupling oxidative phosphorylation. Tl is also known to disrupt normal cell metabolism by stimulating several  $\text{K}^+$ -dependent enzymes, such as phosphatase, homoserine dehydrogenase, vitamin  $\text{B}_{12}$ -dependent diol dehydrogenase, L-threonine dehydratase, and AMP deaminase.

Tl has been demonstrated to increase the levels of hydrogen peroxide and decrease the levels of glutathione, which leads to enhanced lipid peroxidation, oxidative stress, and energy depletion primarily in the brain (Hasan and Ali, 1981; Hanzel and Verstraeten, 2005). Tl-induced changes, such as cellular energy loss, depletion of flavoproteins, and binding of Tl to active sulfhydryl sites of enzymes, are the proposed mechanisms for Tl neuropathy. The complexing of Tl to cysteine appears to inhibit crosslinking of proteins and thereby causes inhibition of keratinization of hair. Tl blocks formation of the disulfide bonds in keratin and thus leads to hair loss (Mulkey and Oehme, 1993). Most evidence supports direct involvement of Tl with the hair follicles as the mechanism of hair loss.

## Toxicity

Tl toxicity has been studied extensively in rats, mice, guinea pigs, rabbits, dogs, and humans. In general, Tl compounds are extremely toxic to humans and animals. Tl has been classified as the most toxic cumulative metal cation. A single dose of 15 mg Tl/kg body weight has been found to be lethal in guinea pigs, dogs, and humans. The  $\text{LD}_{50}$  is 15 mg/kg in rats and 16–27 mg/kg in mice. Of course, these values vary depending on the form of Tl (Mulkey and Oehme, 1993).

Onset of signs and symptoms of Tl poisoning is slow (i.e., within 48h). The signs and symptoms of Tl toxicity (thallotoxicosis) can vary depending on the species, age, form of Tl, dose, and acute versus chronic exposure. Gastroenteritis, polyneuropathy, and hair loss are the dominant features of Tl poisoning (Mulkey and Oehme, 1993). Acutely poisoned animals show abdominal pain, vomiting, and constipation or diarrhea. At a high dose exposure, neurologic signs dominate with peripheral neuropathy. Sensory disturbances include pain, ataxia, and paresthesia, which progress to muscle atrophy. Muscle fibers can show myopathic changes with abnormal central nucleoli, striated transverse fibers disappearance, necrosis, and fibrosis. Cats dosed with Tl at 4 mg/kg body weight developed hypotonia and ataxia mainly due to pathological changes in sensory neurons (Sager, 1994). Dogs and cats exposed to Tl also showed hemorrhagic gastroenteritis and hepatic and renal damage.

Surviving animals usually show signs of alopecia, bloody lesions of the skin, tremors, neuropathy (involving both central and peripheral nervous systems), and paralysis of muscles. In addition, Tl causes structural and functional changes in heart and kidneys. Evidence from experimental animal studies suggests that the reproductive system is highly susceptible to Tl. Humans and animals accumulate Tl in testes, where morphological and biochemical changes occur. Tl is embryotoxic and teratogenic in chick embryo, causing achondroplasia, leg bone curvature, beak deformity, microcephaly, and decreased fetal size. However, teratological investigations in mammals have produced conflicting results (Gregotti and Faustman, 1998). For further details on Tl toxicity, refer to Mulkey and Oehme (1993) and Galvan-Arzate and Santamaria (1998).

### Diagnosis

Diagnosis of Tl poisoning is based on evidence of Tl exposure, clinical signs, and chemical confirmation in body tissue/fluid. Quantitative analysis for Tl in urine, serum, feces, saliva, or hair is performed to assess the extent of Tl exposure and to monitor treatment. Tl residue analysis at diagnostic labs is commonly carried out using atomic absorption spectrometer, inductively coupled plasma (ICP), or ICP-mass spectrometer-based methods. Tl-induced hematological changes may include anemia, leukocytosis, eosinophilia, and lymphocytopenia. Decreased creatinine clearance, elevated BUN, and proteinuria indicate renal function impairment. Alopecia is usually observed 3 or 4 weeks postexposure, thus diminishing the timely treatment.

### Treatment

Therapy for Tl poisoning should be instituted as early as possible. In the case of acute oral ingestion, supportive

care should include induction of vomiting followed by gastric lavage and use of laxatives to remove Tl as much as possible from the GI tract. Prussian blue (potassium ferric hexacyanoferrate (II)) is the treatment of choice for Tl exposure. Prussian blue acts by binding to Tl in the GI tract and makes it unavailable for absorption or reabsorption. Hemodialysis and hemoperfusion can be used to remove Tl from the circulation. At later stage of the treatment, potassium can be used to mobilize Tl from the tissues. Maintenance of vital organ functions should be a priority, especially in acute cases. If Tl poisoning is diagnosed and treated early, the chance for full recovery is good.

### Conclusion

Tl is one of the most toxic heavy metals used as a rodenticide. It produces a wide range of toxic effects involving multiple organs and mechanisms. Neurological, renal, and gastrointestinal effects are of primary concern. Exposure with a high dose is often fatal. Surviving animals often show complex signs of Tl poisoning, especially polyneuropathy, renal impairment, and hair loss. The diagnosis of Tl poisoning is based on clinical signs and Tl detection in urine, serum, or other biological tissue/fluids. Timely therapy with Prussian blue and supportive measures can lead to full recovery.

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## Avitrol

Ramesh C. Gupta

## INTRODUCTION

Avitrol (4-aminopyridine) is a white, odorless, crystalline sand-like material that is readily soluble in water. It has a chemical formula of  $C_5H_6N_2$  with a molecular weight of 94.1. Its chemical structure is shown in [Figure 58.1](#).

4-Aminopyridine was developed by the Phillips Petroleum Company and marketed in 1963 as an avicide under the name “Avitrol.” It was also considered a bird repellent. Currently, it is a product of the Avitrol Corporation (Tulsa, OK, USA). Avitrol has many other names, including 4-AP, 4-pyridylamine, 4-pyridamine, and amino 4-pyridine. Avitrol is currently used to control the overpopulation of certain birds that are considered pest birds, including pigeons, red-winged blackbirds, blackbirds, cowbirds, grackles, sparrows, starlings, gulls, and crows.

Depending on the bird species to be controlled, the formulation can be prepared in grain/corn or bread. Avitrol is available in various formulations, such as grain baits (0.5, 1, and 3%) and powder concentrate or powder mix (25 and 25%). According to the U.S. Environmental Protection Agency (EPA), grain bait formulations of 4-aminopyridine are categorized in toxicity class III, whereas powder concentrate formulations are in toxicity class I. Avitrol is intended to make a few birds in the flock sick; that is, the sick birds thrash around and scare

off the other birds. In higher doses, avitrol can not only be toxic or lethal to birds but also be lethal to non-target species. The use of 4-AP in birds has been criticized by the Humane Society of the United States ([Brasted, 2008](#)). This chapter describes the toxicity of avitrol in birds, mammals, and fish.

## BACKGROUND

Scientists have been seeking an ideal way to control pest birds that destroy crops, feed/grain meant for livestock, and ruin the look of beautiful buildings and monuments. A number of chemicals have been investigated for their ability to repel birds ([Clark, 1998](#); [Dolbeer \*et al.\*, 1998](#); [Stevens and Clark, 1998](#)). Currently, avitrol is the most popular avicide that is registered at the EPA for the control of certain pest birds that feed on cattle feed lots, field corn, wheat, sorghum, sunflowers, peanuts, pecans, grain, feed processing plants, etc. Avitrol is also used to drive away birds from monuments, airports, warehouse premises, and other public buildings ([Thomson, 1991–1992](#)). Upon ingestion, avitrol causes utter vocalization and physical distress (i.e., hyperactivity) in certain birds, which acts as an area repellency to the remainder of the flock – a so-called “flock frightening syndrome” ([Spyker \*et al.\*, 1980](#); [Meister \*et al.\*, 1984](#)). Wildlife feeding on treated bait can also be killed. In recent years, the use of avitrol has been banned in many cities, including New York, San Francisco, and Boulder, Colorado, because of its toxicity to non-target species. Many field cases of avitrol poisoning have been reported in birds and domestic, wild,

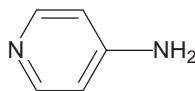


FIGURE 58.1 Chemical structure of 4-aminopyridine.

and zoo animals (Schafer, 1972; Schafer *et al.*, 1974; Nelson *et al.*, 1976; Ray *et al.*, 1978; Spyker *et al.*, 1980; Frank *et al.*, 1981; Nicholson and Prejean, 1981; Smith and Lewis, 1988; Conover, 1994). Avitrol has also been involved in suicidal attempts (Pickett and Enns, 1996; Johnson and Morgan, 2006). During approximately the past decade, 4-AP has received much attention because of its extensive application as a pharmacological drug in neurodegenerative diseases (Davidson *et al.*, 1988; Pinter *et al.*, 1997; Jensen and Shi, 2003) and as experimental potassium channel blocker (Hu *et al.*, 2006), calcium channel activator (Wu *et al.*, 2009), and apoptosis inducer (Chin *et al.*, 1997).

## TOXICOKINETICS

Like other aminopyridines, 4-aminopyridine is rapidly absorbed from the gastrointestinal (GI) tract into circulation. The compound is readily metabolized in the liver, and metabolites are excreted in the urine. Approximately 90% of the administered dose, following intravenous (i.v.) or oral administration, excretes in the urine.

## MECHANISM OF ACTION

In acute toxicity, the central nervous system (CNS) is the target organ, whereas in chronic toxicity the CNS and liver are the target organs. 4-Aminopyridine blocks potassium ion channels and increases acetylcholine levels at the synapses and neuromuscular junctions (Rowan, 1985). This results in hyperactivity, convulsions, and seizures. In acute toxicity, birds and animals suffer immensely. Avitrol impairs the bird's nervous system, and as a result, birds become disoriented and exhibit erratic flight, tremors, and violent convulsions for hours before they finally succumb to death. Avitrol also causes excess methemoglobin formation. Methemoglobin is unable to carry oxygen to the tissues, thereby causing respiratory distress. Death ensues due to cardiac and respiratory arrest.

## TOXICITY

Avitrol is highly toxic to all species of birds. It is also toxic to mammals, humans, fish, and wildlife. The oral acute LD<sub>50</sub> values (expressed as mg/kg body weight) for avitrol are reported to be 10–12 for chickens, 8 for gulls, 3 for crows, 5.6 for pheasants, 15 for bobwhite quail, 8 for mourning dove, 3.8–4 for sparrows, 5–6 for starlings,

4–7 for pigeons, 9 for blackbirds, and 3.2 for boat-tailed grackle (NPCA, 1972). The oral acute LD<sub>50</sub> value in rats is 20 mg/kg (Meister *et al.*, 1984), and in dogs it is 3.7 mg/kg (NPCA, 1972). The dermal LD<sub>50</sub> of technical-grade 4-AP in rabbits is 326 mg/kg (NPCA, 1972). LC<sub>50</sub> values of 4-AP for coturnix quails, mourning doves, and mallard ducks are 447, 316, and 722 ppm, respectively.

Avitrol produces toxicity in birds in a dose-dependent manner; that is, birds exposed to a low dose exhibit utter distress calls, whereas with higher dose they become incapacitated and die. Birds exposed to avitrol usually show onset of signs within 5–15 min and signs of severe intoxication for a period of 30–60 min. Poisoned birds become disoriented and exhibit erratic flight. Eventually, they are unable to stand or sit in a sternal position, and they show tremors and convulsions for a brief period to hours (Conover, 1994). In a case report, Bischoff *et al.* (2001) described the primary signs of poisoning in crows as frequent vocalization, CNS abnormalities, and inability to fly or walk. “Downed” birds either die or recover within 1–15 h (NPCA, 1972). Birds intoxicated with avitrol that react and alarm a flock usually die.

Non-target species including mammals are equally sensitive to the toxicity of avitrol. In mammals, avitrol produces symptoms similar to those of epileptic seizures. Doses near the LD<sub>50</sub> exert a usual sequence of symptoms including hyperexcitability, salivation, tremors, muscle incoordination, and convulsions, and death results from cardiac and respiratory failure. Most of these symptoms are associated with hypercholinergic activity. In general, onset of signs occurs within 10–15 min and death occurs within 15 min to 4 h.

Predators such as raptors, foxes, hawks, cats, and dogs die from secondary poisoning after feeding on dead or dying birds. Secondary poisoning has also been found in endangered birds, including red-tailed hawks and peregrine falcons, as they died from ingesting the remains of pigeons and other birds poisoned with avitrol.

Dermal exposure to avitrol can lead to systemic intoxication or general overall poisoning. Very few chronic studies have been done, and no conclusive findings have been reported. Currently, studies are not available to suggest that avitrol has any potential for mutagenic, teratogenic, or carcinogenic activity.

In fish, avitrol has been found to be moderately toxic (EXTOXNET, 1996). Fish become increasingly sensitive with increased exposure. The LC<sub>50</sub> ranges from 4 mg/L (in soft water) to 2.43 mg/L (in hard water) in channel cat fish. The LC<sub>50</sub> ranges from 3.40 mg (in soft water) to 3.2 mg/L (in hard water) in bluegill.

In humans, avitrol is known to cause seizures. Exposed individuals exhibit weakness, diaphoresis, altered mental status, and hypertension. Other symptoms include thirst, nausea, dizziness, weakness, ataxia, tremors, dyspnea, and tonic-clonic convulsions. Avitrol

also produces metabolic acidosis, leukocytosis, and elevations of serum enzymes (glutamic oxaloacetic transaminase, lactate dehydrogenase, and alkaline phosphatase) as notable laboratory findings. Death occurs due to respiratory failure.

## Diagnosis

Diagnosis of avitrol poisoning is based on history of exposure; clinical signs; pathologic findings; and residue determination of 4-AP in crop, GI content, or tissues. The crop and GI content have the highest levels of 4-AP. Residues of 4-AP can be determined using liquid chromatography (Uges and Bouma, 1981) and may be confirmed by gas chromatography-mass spectrometry. Small residue can also be found in the liver, heart, muscle, kidney, brain, and lung. Residue of 4-AP can be determined by thin layer chromatography, high-performance liquid chromatography, or gas chromatography-mass spectrometry.

## TREATMENT

Pancuronium is a pharmacologic antidote and is recommended in severely poisoned human patients. Propranolol appears to block some of the cardiac toxicity (e.g., cardiac arrhythmias) of 4-aminopyridine. Seizures can be treated with diazepam (0.1 mg/kg, i.v.). In severe cases, phenobarbital or phenytoin can be given if there is no response to diazepam. In the case of avitrol ingestion, general symptomatic and supportive treatment includes emesis, gastric lavage, activated charcoal, and cathartic sodium thiosulfate. Bicarbonate should be added to the fluids to treat acidosis.

## CONCLUSIONS

Avitrol is commonly used to deter pest birds from roosting and nesting. The compound is toxic to all species of birds. It is also toxic to mammals, birds, and fish. Secondary poisoning is very common in non-target birds and dogs. Avitrol produces toxicity by affecting the CNS, and as a result, toxic signs include hyperactivity, convulsions, and seizures.

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# Toxic gases

Rhian Cope

## INTRODUCTION

For the purposes of this chapter, a gas is defined as a state of matter consisting of particles that have neither a defined volume nor a defined shape at standard temperatures and pressures. A vapor represents the gas phase of components from substances that are either solid or liquid at standard temperatures and pressures. Although much of the toxicology of gases is applicable to the toxicology of vapors, the focus of this chapter is classical gas toxicology.

This chapter is divided into two sections: general principles and specific toxic gases. The general principles section covers basic gas toxicokinetics and basic dosimetric adjustments for human risk assessment. The specific toxic gases section covers specific toxic gases that are of veterinary significance as well as those to which veterinarians are likely to be exposed, including carbon monoxide, hydrogen sulfide, oxides of nitrogen (sil-

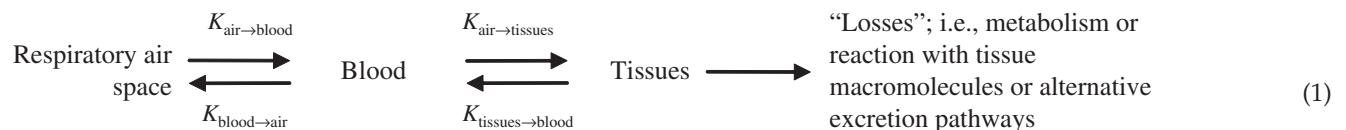
filler's disease), gaseous ammonia (including anhydrous ammonia), and smoke inhalation.

## GENERAL PRINCIPLES

### Toxicokinetics

#### *Absorption and distribution*

Depending on their physicochemical properties, gases can be absorbed throughout the respiratory tract with resultant site of first contact effects, systemic toxicity, or a combination of both. The driving force for the systemic (i.e., entry into the bloodstream) and tissue absorption of inhaled gases is diffusion down concentration gradients (Rozman and Klaassen, 2001; Witschi and Last, 2001; Renwick, 2008; Valentine and Kennedy, 2008). This can be described at a high level by a series of simplified toxicokinetic equilibria and related equilibrium constants ( $K$ ):



The absorption of a gas will continue until equilibria are established in the various compartments. Once the equilibria are established, no net absorption will occur unless there is loss from the system (i.e., metabolism), reaction with tissue molecules, or excretion through nonrespiratory pathways (e.g., renal excretion). It is critical to note that just because the equilibria have been established does not mean that the concentrations in the relevant compartments are all equal. Rather, once the equilibria are established, the concentration ratios between the various compartments will remain constant provided that saturation of the system does not occur – that is, the air:blood:tissue(s) concentration ratios remain constant.

Absorption of the gas will continue if “losses” from the system occur. If the substance is lost through metabolism, the rate of continued absorption once the equilibria have been established is effectively the rate of metabolism of the substance. This mass-balance relationship is essentially the basis of closed respirometry measures of oxygen consumption and metabolic rate (that were pioneered by Laplace and Lavoisier in the 1770s). If the substance is lost from the system solely through nonrespiratory excretion once the equilibria have been established, the rate of continued absorption is essentially equivalent to the rate of nonrespiratory excretion.

Also, if the concentration of the gas in the tissue(s) is higher than that in the respiratory airspaces, the net diffusive force will drive the movement of the gas from the tissue(s) into the air. An example is the excretion of carbon dioxide produced by aerobic metabolism in the tissues that is driven by simple diffusion. This is also the basis for the net excretion of a gas once exposure has ceased.

In normal circumstances, gas diffusion is not rate limiting for the systemic absorption for three basic reasons: (1) generally ionized, hydrophilic molecules that are apt to be diffusion limited across biological membranes have low volatility and thus their concentration in normal air is very low; (2) alveolar type I pneumocytes are very thin and are in intimate contact with the pulmonary capillaries, effectively resulting in extremely short diffusion distances; and (3) gases absorbed through the alveoli are rapidly removed by the blood (Rozman and Klaassen, 2001; Witschi and Last, 2001; Renwick, 2008; Valentine and Kennedy, 2008). There are two exceptions: (1) carbon monoxide absorption is diffusion limited (the basis for its use in the lung diffusion capacity test or determination of the DLCO which is referred to as the TLCO or transfer capacity in Europe), and (2) the presence of interstitial lung disease increases the pulmonary diffusion distances and results in diffusion-limited absorption for gases (notably oxygen).

In a broad sense, two innate substance characteristics affect the rate of establishment of the equilibrium

between the respiratory airspaces and the bloodstream: (1) whether or not uptake of the gas is perfusion limited and (2) whether or not the uptake of the gas is ventilation limited (Rozman and Klaassen, 2001; Witschi and Last, 2001; Renwick, 2008; Valentine and Kennedy, 2008). These characteristic factors are directly related to a physicochemical property of the relevant gas called the blood:gas partition coefficient. This is the solubility ratio for the chemical in gas phase and the chemical dissolved in blood at equilibrium. For substances with low blood:gas partition coefficients, the ability of blood to absorb the gas is rapidly saturated, and thus only a small amount of the gas in the respiratory tract is removed by blood during each circulation. In such circumstances, changes in respiratory rate and/or respiratory minute volume have relatively little effect on the rate of transfer of the gas from the respiratory system to the bloodstream. However, small changes in the rate of flow of blood through the respiratory tract (i.e., perfusion) have relatively large effects on the rate of transfer of the gas from the respiratory system to the blood; that is, increasing pulmonary perfusion results in more rapid removal of the gas from the site of equilibration (the alveolar membranes) and thus a more rapid rate of transfer of the gas from the respiratory system to the bloodstream. Thus, for gases with a low blood:gas partition coefficient, blood gas equilibration occurs relatively quickly (~8–21 min for relatively blood-insoluble gases), and the absorption of such gases is essentially perfusion limited. Perfusion-limited gases tend to be sparingly soluble in water, relatively lipophilic and hydrophobic, and have high octanol:water partition coefficients (Fiserova-Bergerova and Diaz, 1986). However, other factors, such as the affinity of the particular gas to protein and other elements in blood, may also significantly impact the blood:gas partition coefficient (Veltman *et al.*, 2009). Notably, alteration in the level of lipid components in the blood (particularly blood triglycerides) can significantly increase the blood:gas partition coefficient of lipophilic xenobiotics and thus affect their inhalation absorption kinetics (Lin *et al.*, 2002). Ingestion of a high-fat meal during or before inhalation may result in an increased absorbed dose of an inhaled lipophilic gas, as well as affect the rate of excretion by exhalation of such substances.

The alternate extreme is gases that are ventilation limited ((Rozman and Klaassen, 2001; Witschi and Last, 2001; Renwick, 2008; Valentine and Kennedy, 2008). Ventilation-limited gases are highly soluble in blood (they tend to be hydrophilic or, in rare cases, zwitterion-like) and often have low octanol:water partition coefficients (Fiserova-Bergerova and Diaz, 1986). Again, other factors, such as the affinity of the particular gas to protein and other elements in blood, may also significantly impact the blood:gas partition coefficient (Veltman *et al.*,

2009). The majority of a ventilation-limited gas present at the respiratory exchange site is removed during each respiratory cycle because of its high affinity for blood. Thus, relatively little of the material remains in the alveoli at the end of each breathing cycle, and replenishment of the gas in the gas-exchange regions is dependent on subsequent ventilation. In this situation, relatively small changes in respiratory minute volume can produce relatively large effects on the rate of transfer of the gas from the respiratory system to the blood. The rate of pulmonary blood perfusion has little effect on the rate of transfer of the gas from the respiratory system to the blood because virtually all of the gas is removed from the gas-exchange site during each circulation, and thus increasing the rate of blood flow through the lung during each circulation would not result in additional gas absorption. The time to blood:gas equilibration for gases that are ventilation limited is at least 1 h and often much longer (days or, in some cases, never).

Gases may also be absorbed in the upper respiratory tract, particularly the nasal cavity (Rozman and Klaassen, 2001; Witschi and Last, 2001; Renwick, 2008; Valentine and Kennedy, 2008; Morris and Buckpitt, 2009). In these cases, gases may be systemically reversibly absorbed via diffusion processes in which the blood:tissue:air partition equilibria (and associated coefficients) are important; alternatively, the gas may react with tissue macromolecules in the upper respiratory tract (including being metabolized). In general, gases that are absorbed in the upper respiratory tract tend to be highly soluble in aqueous solutions (particularly in the mucous lining of the upper respiratory tract), highly reactive (i.e., site of first contact toxicants), or both (Renwick, 2008). For these gases, the nasal cavity can be regarded as being analogous to a “gas scrubber” that prevents or limits the exposure of the deeper respiratory tract tissues. Again, in general, gases that are poorly water soluble are generally poorly absorbed in the upper respiratory tract. A further general rule is that relatively little gas absorption occurs in the conducting airways (anatomic dead space) areas of the respiratory tract.

## Metabolism

Metabolism of gases can occur locally in the respiratory tract or at other distant sites (discussed elsewhere in this book). As a generalization, biotransformation of gases within the respiratory tract primarily occurs at two main locations: within the epithelia of the nasal cavity and within the Clara cells of the lung (Bogdanffy *et al.*, 1986, 1987; Castranova *et al.*, 1988; Baron and Voigt, 1990; Bogdanffy, 1990; Keller *et al.*, 1990; Hukkanen *et al.*, 2002; Castell *et al.*, 2005). Phase I biotransformation also occurs to a lesser degree in type II pneumocytes and in pulmonary macrophages. The immunohistochemical distribution and biochemical activity of biotransformation enzymes in the rat nasal cavity are shown in Table 59.1. As can be seen from the table, biotransformation potential is predominantly located in the olfactory and respiratory epithelia of the nasal cavity. Whether or not metabolism in the olfactory or respiratory epithelium predominates depends on which enzymes are involved and, in some cases, the particular substrate. Although it is notable that both phase I and phase II biotransformation reactions can occur in the nasal cavity, it is also notable that the biotransformation capacity of the nasal cavity is also a prime source of local toxication of xenobiotics (Bogdanffy, 1997).

Numerous studies have demonstrated that Clara cells are a rich source of cytochrome P450-dependent mixed-function oxidases and a prime site for P450-mediated phase I metabolism and toxication in the lung (Devereux, 1984; Devereux *et al.*, 1985; Baron and Voigt, 1990; Hukkanen *et al.*, 2002; Castell *et al.*, 2005).

## Excretion of gases via the respiratory tract

Simple single phase (or single compartment excretion kinetics) excretion of inhaled gases through the respiratory tract is in many ways dependent on the same factors as gas absorption by inhalation. Gases with a high blood:gas partition coefficient tend to be excreted relatively slowly due to the relatively large amount of the gas dissolved in the blood volume and the high affinity

TABLE 59.1 Distribution of biotransformation enzymes in the rat nasal cavity

Tissue type	AldH	Carb	FdH	NBE	P450 red	P450	EH	GSHt	B $\alpha$ POH
Squamous epithelium	Low	No data	No data	Low	No data	No data	No data	No data	No data
Respiratory epithelium	High	High	Present	Moderate	Present	Present	Present	Present	Present
Seromucous glands	No data	No data	No data	Low	Present	Present	Present	Present	Present
Olfactory sustentacular cells	Absent	Present in olfactory mucosa	Present	Moderate	Present	Present	Present	Present	Present
Olfactory nerves	Absent		Present	Absent	Absent	Absent	Absent	Absent	Absent
Bowman's glands	Low		Present	High	Present	Present	Present	Present	Present

AldH, acetaldehyde dehydrogenase; Carb, carboxylesterase; FdH, formaldehyde dehydrogenase; NBE, alpha-naphthyl butyrate esterase; P450 red, NADPH-cytochrome P450 reductase; P450, cytochrome P450s; EH, epoxide hydrolase; GSHt, glutathione-S-transferases; B $\alpha$ POH, benzo(a)pyrene hydroxylase. Data from Bogdanffy *et al.* (1986, 1987, 1991), Bogdanffy (1990), Keller *et al.* (1990), Trela and Bogdanffy *et al.* (1991a,b), and Bogdanffy and Taylor (1993).



of the gas for blood relative to its affinity for air. Gases with a low blood:gas partition coefficient tend to be excreted relatively rapidly because of the relatively small amount present in the blood volume and the greater affinity of the gas for air than for blood. However, respiratory excretion of gases that have a high affinity for tissues, particularly adipose tissues, may be biphasic (have two compartment excretion kinetics). This is most commonly observed with lipophilic gases that have a low blood:gas partition coefficient. The initial phase of excretion is relatively rapid due to the fast removal of the gas from the blood during exhalation. This is followed by a slower phase as the gas slowly redistributes from the tissue (most often adipose tissues) into the bloodstream, from which it is subsequently removed by exhalation.

### Basic dosimetric adjustments for human risk assessment

For the purposes of risk assessment, absorption dose adjustment (from animal data to humans) – that is, calculation of the human equivalent concentration (HEC) – gases can be categorized into three general classes (U.S. EPA, 2009):

- Category 1 gases: These gases are highly water soluble and/or irreversibly reactive in the surface liquid/tissues of the extrathoracic and tracheobronchial regions of the respiratory tract. Examples include hydrogen fluoride, chlorine, and formaldehyde. The following equations are used for calculation of regional gas dose ratios (RGDRs) and HECs:

$$\text{HEC}_{\text{relevant region (extrathoracic or tracheobronchial or pulmonary regions)}} = \text{NOAEC}_{\text{animal}} (\text{mg}/\text{m}^3) \times \text{RGDR} \quad (2)$$

where RGDR is calculated by

$$\text{RGDR}_{\text{extrathoracic regions}} = \frac{\left( \frac{V_E}{SA_{ET}} \right)_{\text{Animal}}}{\left( \frac{V_E}{SA_{ET}} \right)_{\text{Human}}}$$

$$\text{RGDR}_{\text{tracheobronchial region}} = \frac{\left( \frac{V_E}{SA_{TB}} \right)_{\text{Animal}}}{\left( \frac{V_E}{SA_{TB}} \right)_{\text{Human}}}$$

$$\text{RGDR}_{\text{pulmonary region}} = \frac{\left( \frac{Q_{Alv}}{SA_{PU}} \right)_{\text{Animal}}}{\left( \frac{Q_{Alv}}{SA_{PU}} \right)_{\text{Human}}}$$

Definitions:

- HEC = human equivalent concentration at the extrathoracic site of action
- NOAEC<sub>Animal</sub> = no observable adverse effect concentration for extrathoracic effects or tracheobronchial effects (or a similar toxicological threshold or dose)
- RGDR = regional gas dose ratio
- V<sub>E</sub> = respiratory minute volume in mL/minute
- SA<sub>ET</sub> = surface area of the extrathoracic region in cm<sup>2</sup>
- SA<sub>TB</sub> = surface area of the tracheobronchial region in cm<sup>2</sup>
- SA<sub>PU</sub> = surface area of the pulmonary region in cm<sup>2</sup>
- Q<sub>Alv</sub> = alveolar ventilation rate mL/min which can be approximated by 0.6 × V<sub>E</sub>

Note: standard values for V<sub>E</sub>, SA<sub>ET</sub>, and SA<sub>TB</sub> can be found in the U.S. EPA 2009 reference and in Derelanko MJ and Hollingeer MA (2001) Handbook of Toxicology, Second Edition. CRC Press.

- Category 3 gases: These gases are relatively insoluble in water and are not reactive in the extrathoracic and tracheobronchial regions of the respiratory tract. These gases are not “scrubbed out” in the upper respiratory tract and conducting airways and thus penetrate into the deep pulmonary areas, where they are available for absorption into the systemic circulation. Examples include styrene and most of the common anesthetic gases and vapors. The following equations used for calculation of an RGDR and HECs:

$$\text{HEC}_{\text{systemic effects}} = \text{NOAEC}_{\text{animal}} (\text{mg}/\text{m}^3) \times \text{RGDR} \quad (3)$$

where RGDR is calculated by

$$\text{RGDR}_{\text{systemic, category 3}} = (H_{b/g})_{\text{Animal}} / (H_{b/g})_{\text{Human}}$$

where  $H_{b/g}$  is the blood:gas partition coefficient.

- Category 2 gases: These gases are moderately water soluble, rapidly and reversibly reactive, and/or moderately to slowly irreversibly metabolized within respiratory tissues. These intermediate gases have the potential for both sites of contact and systemic toxic effects. An example is sulfur dioxide.

Risk assessment methodologies also commonly use Haber’s law for dosimetric adjustments pertaining to the duration of exposure to a gas. Ernest Warren and Fritz Haber noted during their studies of the acute (exposure durations between 1 and 120 min) lethality effects of poison gases (specifically gas weapons in the case of Fritz Haber, notably phosgene, methylchloroformate, cyanide gas, chloroacetone, xylybromide, and chlorine) that exposure to a low concentration of a poisonous gas for a long time often had the same effect (death) as exposure to a high concentration for a short time. Generally, a simple

mathematical relationship applied:  $C \times t = k$ , where  $C$  is the concentration of the poisonous gas,  $t$  is the time of exposure, and  $k$  is a constant or “toxic load” (Haber, 1924; Lohs, 1990; Witschi, 1999, 2000). If different concentrations and times of exposure are used, this implies that  $C_1 \times t_1 = C_2 \times t_2$ . This relationship, in theory, can also be used to extrapolate concentration values between short-term and long-term exposures:

$$C_2 = \frac{C_1 \times t_1}{t_2} \quad (4)$$

In modern risk assessment, the ten Berge modification of Haber’s law is commonly used:  $C^n \times t = k$  (ten Berge *et al.*, 1986). The exponential  $n$  is a regression coefficient for the exposure concentration–exposure duration relationships for the relevant effect. In general, the value of  $n$  lies between 1 and 3. If suitable data are not available to derive  $n$ , a default value of  $n = 1$  is used for extrapolating from shorter to longer exposure durations and a default value of  $n = 3$  is used for extrapolating from longer to shorter exposure durations. Using the ten Berge modification, the Haber’s law equation becomes

$$C_2 = \frac{C_1^n \times t_1}{t_2} \quad (5)$$

However, note that there are many cases in which Haber’s law and the ten Berge modified Haber’s law do not accurately describe the dose–time relationships for the toxicological effects of gases. The use of these simple relationships may seriously over- or underestimate the degree of toxicological effects, particularly when there are large extrapolations in terms of the time of exposure (Weller *et al.*, 1999; Miller *et al.*, 2000; Hoyle *et al.*, 2010). High-quality data for the specific duration of exposure of interest are preferable to the use of Haber’s law or the ten Berge modification.

If the use of Haber’s law (ten Berge modification), respiratory absorption, and the relative volume of air inhaled per unit time are factored into the evaluation, the HEC equation for repeated inhalation exposure becomes

$$\begin{aligned} \text{HEC} = & \text{NOAEC}^n \text{Animal (mg/m}^3) \times \frac{\text{Hours of exposure per day}_{\text{Animal}}}{\text{Hours of exposure per day}_{\text{Human}}} \\ & \times \frac{\text{Days of exposure/week}_{\text{Animal}}}{\text{Days of exposure/week}_{\text{Human}}} \times \frac{\text{Inhaled volume per day}_{\text{Animal}}}{\text{Inhaled volume per day}_{\text{Human}}} \\ & \times \text{RGDR} \end{aligned}$$

where:

$n$  = ten Berge exponential factor;

Hours of exposure per day<sub>Animal</sub> is commonly 4 hours per day for a single acute dose study or 6 hours per day for a repeat dose study

Hours of exposure per day<sub>Human</sub> is commonly 8 hours per day for workers and 24 hours per day for the general population

Days of exposure per week<sub>Animal</sub> is commonly 5 days per week, but up to 7 days per week may be used in some cases

Days of exposure per week<sub>Human</sub> is commonly 5 days per week for workers and 7 days per week for the general population

Inhaled volume per day<sub>Animal</sub>: standard respiratory volumes for rats are 0.29 m<sup>3</sup>/kg for a 6 hour exposure/day, 0.38 m<sup>3</sup>/kg for an 8 hour exposure/day; and 1.15 m<sup>3</sup>/kg for a 24 hour/day exposure. Typically the 6 hour exposure/day value is used for most rat studies.

Inhaled volume per day<sub>Human</sub>:

standard respiratory volume for humans (sedentary) is 5 m<sup>3</sup> for a 70 kg person exposed for 6 hours

standard respiratory volume for humans (sedentary) is 6.7 m<sup>3</sup> for a 70 kg person exposed for 8 hours

standard respiratory volume for humans (sedentary) is 20 m<sup>3</sup> for a 70 kg person exposed for 24 hours

standard respiratory volume for a human worker (light activity) is 10 m<sup>3</sup> for a 70 kg person exposed for 8 hours

## SPECIFIC TOXIC GASES

### Carbon monoxide

#### Overview, uses, and sources of exposure

Carbon monoxide (CO) is colorless, odorless and virtually undetectable without the use of gas detection technologies, hence its reputation as a “silent killer” (Weaver, 2004). CO is ubiquitous and most commonly produced by incomplete hydrocarbon combustion. A component of CO poisoning is almost always present in cases of smoke inhalation injury (Alarie, 2002; Jones, 2003; Fitzgerald *et al.*, 2006). CO is also produced by the mixed function oxidase-mediated biotransformation of methylene chloride (dichloromethane), a common solvent component present in paint strippers and degreasers (Weaver, 2004). The combustion of methylene chloride produces phosgene. CO is also produced endogenously as a byproduct of erythropoiesis.

CO is a very common, high-mortality cause of human poisoning in the United States (Sadovnikoff *et al.*, 1992; Meredith, 1993; McGuigan, 1999; Hampson and Stock, 2006; Harduar-Morano and Watkins, 2011). Epidemics of CO poisoning in humans (and animals) are notoriously associated with the occurrence of storms, particularly when storms are accompanied by a loss of electrical power. Veterinarians, farm workers, and animals are at risk of exposure to CO in intensive animal production

units that are heated using hydrocarbon combustion devices. Both acute lethal and sublethal CO poisoning are well-known problems in intensive pig operations, particularly those relying on gas heating systems (Boller, 1976; Keller, 1976; Wood, 1979; Stuart and Oehme, 1982; Dominick and Carson, 1983; Morris *et al.*, 1985a,b; Pejsak *et al.*, 2008). CO in engine fumes may also reduce egg hatchability (Swarbrick, 1989).

CO has also been investigated as a veterinary euthanasia agent, and it may have some value when mass euthanasia of poultry is necessary (Moreland, 1974; Simonsen *et al.*, 1981; Chalifoux and Dallaire, 1983; Lambooy *et al.*, 1985; Enggaard Hansen *et al.*, 1991; Kingston *et al.*, 2005; Gerritzen *et al.*, 2006). However, there are substantial operator safety concerns with the use of CO for this purpose. CO treatment has also been used to improving the color of muscle foods (Hamling *et al.*, 2008; Mantilla *et al.*, 2008; Jeong and Claus, 2010, 2011). Again, strict safety standards are necessary to protect human workers in such circumstances.

### Toxic dose

At physiological equilibrium, an atmospheric CO level of 50ppm produces a carboxyhemoglobin (COHb) level of 8% in humans, which is the basis for the U.S. Occupational Safety and Health Administration PEL 8-h time-weighted average level of 50ppm (Weaver, 2004). Reduction in cognitive performance occurs in humans exposed to levels as low as 17ppm for 1.5–2.5h (COHb level of 2%). Situations that result in lower alveolar oxygen partial pressure (e.g., high altitudes), increased alveolar ventilation (e.g., higher metabolic rates and increased activity), pre-existing cardiovascular or cerebrovascular disease, cardiac insufficiency, increased affinity of hemoglobin (Hb) for CO (e.g., fetal Hb), and decrease blood oxygen carrying capacity will reduce the tolerance to CO (Weaver, 2004). CO exposure is a noted cause of angina in humans with pre-existing cardiovascular disease.

Exposure of pregnant sows to CO levels of 150–400ppm for 48–96h results in stillbirth rates of 6.7–80.0% (Dominick and Carson, 1983). The risk of stillbirth increases significantly when COHb concentration is greater than 23%. Notably, piglets that are born live in such circumstances usually have hypoxic ischemic leukoencephalopathy. There is evidence that preweaning pigs have some capacity to adapt to high air CO levels given that exposure to 200ppm of CO for the first 21 days of life has no adverse effect on any performance or behavioral characteristics in weanling pigs; however, exposure to 300ppm in such circumstances results in reduced growth and production performance (Morris *et al.*, 1985a,b). Perinatal exposure to 250ppm of CO resulting in a COHb level of approximately 20% produced detrimental effects in neonatal pigs.

### Toxicokinetics and toxicodynamics

CO is one of the few diffusion-limited gases during respiratory absorption. Despite this, CO is rapidly absorbed through the respiratory tract (Weaver, 2004). Approximately 85% of absorbed CO binds to Hb with an affinity approximately 200–300 times higher than that of oxygen. The remainder binds to myoglobin in muscle and to blood proteins. Very small amounts are metabolized to carbon dioxide, which is subsequently exhaled. CO is excreted primarily through the respiratory tract and has a whole body half-life ( $t_{1/2}$ ) of 3 or 4h in adult humans.

Treatment with 100% oxygen shortens the adult human CO  $t_{1/2}$  to approximately 30–126min, and treatment with hyperbaric oxygen further shortens this to 23min (Weaver, 2004). The human COHb  $t_{1/2}$  is much longer at 7h.

### Pathophysiology

The principal mode of action of CO is tissue hypoxia secondary to reduced blood oxygen carrying capacity (Weaver, 2004). Reduced blood oxygen carrying capacity occurs because of the preferential binding of CO rather than oxygen to Hb (the affinity of Hb for CO is approximately 200–300 times higher than that of oxygen). The presence of COHb also results in a left shift of the oxygen:Hb dissociation curve, further exacerbating tissue hypoxia. Organs and tissues with poorly developed anastomotic vessels and high metabolic rates (e.g., the heart and brain) are particularly susceptible. Venous blood, which has a high level of COHb, is described as being “cherry red” in color.

Myoglobin also has a higher affinity for CO compared with oxygen (Weaver, 2004). Cardiac myoglobin is particularly vulnerable to this effect, and the result is direct myocardial depression. CO–myoglobin is described as having a bright pink to red coloration. CO also binds to cytochrome oxidases *in vitro* although at lower affinity than oxygen. Thus, the *in vivo* relevance of this effect is uncertain. However, such metabolic effects may be important under conditions of tissue hypoxia.

CO produces substantial endovascular oxidative stress (Weaver, 2004). CO triggers the release of oxygen radicals from neutrophils and triggers nitric oxide release from platelets with the subsequent formation of peroxynitrate. CO also triggers brain neuronal apoptosis, particularly in the hippocampus, which contributes to the common amnesic effect of the gas.

### Vulnerable populations

Vulnerable populations include any life stage in which fetal Hb is present, pregnant individuals, and individuals with pre-existing cardiovascular disease (Weaver, 2004).

### *Clinical presentation*

The most common clinical presentation is that the animal is found either unconscious or dead following acute high-level exposure. Nonpigmented mucous membranes, nails, and skin may have “cherry red” color. Humans with CO poisoning have been described as looking “pink-cheeked and healthy” (Weaver, 2004). Unfortunately, cherry red coloration of the mucous membranes, skin, and nails is not a reliable diagnostic sign, and often “cherry red means dead.” When CO is used as a euthanasia agent in dogs, short periods of vocalization and agitation can occur, even when the animal is apparently unconscious (Chalifoux and Dallaire, 1983).

With nonlethal exposures, any body system, organ, or tissue can be affected (Weaver, 2004). Most commonly, clinical signs pertain to hypoxic central nervous system (CNS) damage and may include apparent weakness, fatigue, depression, transient loss of consciousness, and seizure disorders. Cardiovascular signs and effects usually pertain to the effects of reduced blood oxygen carrying capacity, tissue hypoxia, and direct effects on the myocardium and may include exercise intolerance, dyspnea, syncope, and cardiac arrhythmias. Other noted effects include retinal hemorrhage, hearing loss, rhabdomyolysis, peripheral neuropathies, vomiting, and nausea. Permanent CNS injury, particularly to the hippocampus, caudate nucleus, globus pallidus, and the substantia nigra bilaterally, as well as the cerebellum, cerebral cortex, and dorsal thalamus, can be expected in animals that survive significant CO poisoning (Kent *et al.*, 2010).

In pigs, the most common clinically observable effects are abortion storms, stillbirth, increases in perinatal mortality, and reduced neonatal growth rates (Wood, 1979; Dominick and Carson, 1983; Morris *et al.*, 1985a,b). Reduced hatching rates may be observed in poultry (Swarbrick, 1989). Critically, human workers in these facilities may also experience symptoms of CO poisoning, the most common of which are headache, nausea, dizziness, vomiting, and weakness.

### *Diagnostic testing*

Elevated blood COHb is the most definitive form of testing (Weaver, 2004). The presence of COHb cannot be determined by normal pulse oximetry because these instruments cannot discriminate between COHb and oxyhemoglobin. Pulse co-oximetry is necessary. The presence of fetal Hb may give falsely elevated COHb levels during pulse co-oximetry. Electrocardiography is recommended because of the likely presence of cardiac arrhythmias and myocardial damage. Computerized tomography (CT) and magnetic resonance imaging of the brain may reveal brain neurological injury (particularly in the globus pallidus and other basal ganglia and the hippocampus); however, CT images may be normal in the early stages of poisoning.

### *Postmortem findings*

Common gross lesions include cherry red discoloration of the subcutaneous tissues, muscle, blood, and viscera. Unlike with cyanide poisoning, the cherry red discoloration does not fade relatively quickly over time. Histopathology findings include focal leukoencephalomalacia and other lesions that resemble brain ischemic hypoxia, particularly in the basal ganglia (especially the globus pallidus), cerebral cortex, and cerebral white matter.

### *Treatment*

The immediate treatment priority is the prompt removal of the patient from the source of exposure. Rescuers need to take great care not to become casualties in such circumstances. The administration of 100% oxygen may be helpful because this reduces the CO  $t_{1/2}$ . Hyperbaric oxygen therapy may offer some additional benefits, but it is rarely available in veterinary practice (Stoller, 2007).

### *Prognosis*

The prognosis is often poor. Although few data are available from the veterinary literature, human data indicate that a high proportion of survivors develop long-term or permanent neurological, neurocognitive, and neuropsychiatric sequelae (Hampson *et al.*, 2001; Weaver, 2004; Hopkins and Woon, 2006; Tapeantong and Pongvarin, 2009).

### *Prevention*

The best form of prevention is the effective use of CO monitors and alarms in addition to well-adjusted heating equipment and good ventilation.

## **Hydrogen sulfide**

### *Overview, uses, and sources of exposure*

Note that polioencephalomalacia in ruminants due to excessive intakes of sulfur (which is converted to hydrogen sulfide by rumen microflora-mediated metabolism) is discussed in other chapters of this textbook. Within the veterinary context, hydrogen sulfide ( $H_2S$ ; “sewer gas,” “swamp gas,” “sour gas,” and “stink damp”) is most commonly encountered as a byproduct of the decomposition of sulfur-containing organic material, particularly with manure tanks, septic tanks, sludge pits, cesspools or settling ponds, or enclosed spaces containing decomposing feed (Caravati, 2004; Chou *et al.*, 2006).  $H_2S$  may also be produced when hydrochloric acid and/or sulfuric acid are used to clean drains or when wool, hair, and hides are burnt.  $H_2S$  is also a byproduct of petroleum refining, tanning, rubber vulcanizing, coal and gas production, and a number of other industrial processes.  $H_2S$  is colorless, heavier than



TABLE 59.2 Dose thresholds for hydrogen sulfide

Concentration (ppm)	Effect
0.02	Human odor threshold
10	Obvious unpleasant odor + mild eye irritation
20	U.S. OSHA PEL 15-min ceiling limit
50	Definite conjunctival irritation
50–100	Mild respiratory irritation
100	Olfactory fatigue
150–200	Olfactory nerve paralysis
250	Prolonged exposure results in pulmonary edema
300–500	Imminent threat to life plus pulmonary edema plus potentially apnea
500	30- to 60-min exposure results in excitement, staggering, unconsciousness, apnea, and respiratory failure
500–1000	Acts primarily as a systemic poison producing unconsciousness and death due to respiratory paralysis
700	Rapid unconsciousness and death if not rescued immediately
5000	Sudden death

Data from Beauchamp *et al.* (1984).

air (concentrates in low-lying areas), flammable, and explosive.

H<sub>2</sub>S is a common and important cause of sudden death in humans, and carries with it the mantra “one breath means death” (Caravati, 2004). Within the farm context, agitation of solutions containing dissolved H<sub>2</sub>S may greatly increase the level in air (Hooser *et al.*, 2000). This is of great importance in the case of manure tanks, septic tanks, sludge pits, and settling ponds. Exposure of confined cattle to H<sub>2</sub>S liberated by agitation of a manure pit has resulted in significant mortality. A very common cause of human casualties due to H<sub>2</sub>S poisoning involves misguided rescue attempts (referred to as the “H<sub>2</sub>S lemmings over the cliff phenomenon”).

**Toxic dose**

Relevant dose thresholds for H<sub>2</sub>S are summarized in Table 59.2. A critical point in the dose response is the onset of odor fatigue and paralysis of the olfactory nerves (Caravati, 2004). At this point, individuals can no longer readily detect that they are being exposed to H<sub>2</sub>S.

**Toxicokinetics and toxicodynamics**

H<sub>2</sub>S is rapidly absorbed through the lungs, although respiratory excretion is minimal (Caravati, 2004; Chou *et al.*, 2006). Metabolic detoxification to sulfate within

erythrocytes and hepatocyte mitochondria occurs relatively rapidly. Sulfate is primarily eliminated in urine. Approximately 85% of an acutely lethal dose is eliminated per hour.

**Pathophysiology**

The classical pathophysiology is essentially the same as that for cyanide poisoning (Reiffenstein *et al.*, 1992; Smith, 1997; Milby and Baselt, 1999; Albin, 2000; Caravati, 2004; Woodall *et al.*, 2005; Chou *et al.*, 2006; Khoshniat, 2008; Oesterhelweg and Puschel, 2008; Ballerino-Regan and Longmire, 2010). H<sub>2</sub>S binds to the ferric moiety of cytochrome *c* oxidase, thus disrupting the mitochondrial electron transport chain, resulting in blocking of cellular aerobic energy generation. Anaerobic metabolism then predominates, resulting in lactate accumulation and metabolic acidosis. At lower concentrations, H<sub>2</sub>S is an eye, mucous membrane, and respiratory irritant (Caravati, 2004). The respiratory effects of H<sub>2</sub>S (initial respiratory stimulation followed by apnea) are produced by direct central respiratory effects and/or by sulfide stimulation of the chemoreceptors of the carotid body, which results in initial respiratory hypocapnia followed by reflex apnea that may be sustained and/or lethal if significant hypocapnia is present (Beauchamp *et al.*, 1984). Death from H<sub>2</sub>S poisoning is usually due to respiratory arrest and hypoxia (Caravati, 2004). Individuals who survive the initial exposure may subsequently die from the effects of pulmonary edema.

**Vulnerable populations**

Fetuses and neonates are assumed to be more vulnerable than adults (Caravati, 2004).

**Clinical presentation**

The H<sub>2</sub>S knockdown or “one breath means death” phenomenon occurs with exposures greater than 750ppm and is characterized by sudden collapse, loss of consciousness, and apnea (Reiffenstein *et al.*, 1992; Smith, 1997; Milby and Baselt, 1999; Albin, 2000; Caravati, 2004; Woodall *et al.*, 2005; Chou *et al.*, 2006; Khoshniat, 2008; Oesterhelweg and Puschel, 2008; Ballerino-Regan and Longmire, 2010). Individuals may recover if exposure ceases, although permanent neurological damage is a common sequel. Acute respiratory distress due to pulmonary edema occurs following prolonged exposures to greater than 250ppm (Caravati, 2004). At lower levels of exposure, upper respiratory and ocular irritation effects may dominate the clinical picture. Reactive airway disease, bronchiolitis obliterans, and pulmonary interstitial fibrosis have been reported in humans with chronic irritant exposures. Cardiac arrhythmias, nausea, vomiting, diarrhea, and abdominal pain are also common.

### Diagnostic testing

Measurements of H<sub>2</sub>S metabolites such as sulfide and thiosulfate in whole blood and/or urine, as well as breathing zone air sampling/monitoring, are useful (Caravati, 2004).

### Postmortem findings

Greenish discoloration of gray matter, viscera, and bronchial secretions may be evident in fresh tissues, but the coloration is lost with formalin fixation (Park *et al.*, 2009). Fresh tissues may also have a distinctive sulfide smell and may undergo accelerated decomposition.

### Treatment

The immediate treatment priority is the prompt removal of the patient from the source of exposure (Reiffenstein *et al.*, 1992; Smith, 1997; Milby and Baselt, 1999; Albin, 2000; Caravati, 2004; Woodall *et al.*, 2005; Chou *et al.*, 2006; Khoshniat, 2008; Oesterhelweg and Puschel, 2008; Ballerino-Regan and Longmire, 2010). Rescuers need to take great care not to become casualties in such circumstances. The next treatment priority is resuscitation and provision of 100% oxygen (Caravati, 2004). Induction of methemoglobinemia by administration of nitrites, based on the predilection of sulfide to bind to ferric ions forming sulfmethemoglobin and thus potentially removing the sulfide from cytochrome oxidase and reactivating aerobic metabolism, has been suggested as an antidotal therapy. In clinical reality, nitrites are only effective if administered within seconds to minutes of exposure. Hydroxycobalamin has been proposed as an antidote for H<sub>2</sub>S poisoning on the basis that the pathophysiology resembles that of acute cyanide poisoning (Truong *et al.*, 2007; Fujita *et al.*, 2011). This antidote seems to have some effect under laboratory conditions. However, its effectiveness under field conditions has not been adequately investigated. Hyperbaric oxygen has also been proposed as being beneficial; however, it is rarely available in veterinary practice (Smilkstein *et al.*, 1985; Whitcraft *et al.*, 1985; Lindenmann *et al.*, 2010).

### Prognosis

The prognosis is variable depending on the duration of apnea and the degree of CNS hypoxia (Caravati, 2004). Some individuals appear to recover without any subsequent problems; however, a significant proportion of survivors will develop subsequent neurological problems associated with brain hypoxia.

### Prevention

Loss of detection ability (odor fatigue and/or olfactory nerve paralysis), ill-advised entries into closed spaces, and

misguided attempts to rescue casualties have all been associated with many deaths. The use of electronic exposure monitors (both personal and in spaces) and the immediate availability of personal protective equipment have saved many lives. Great care should be taken not to stir or agitate manure tanks, septic tanks, sludge pits, cesspools, or settling ponds. The use of hydrochloric acid and/or sulfuric acid-based drain cleaners should be avoided.

## Oxides of nitrogen (silo filler's disease)

### Overview, uses, and sources of exposure

The main gas involved is nitrogen dioxide (NO<sub>2</sub>), although other reactive oxides of nitrogen may also be present (Lowry and Schuman, 1956; Jonas, 1984; Pladson, 1984; Douglas *et al.*, 1989; Epler, 1989; Gurney *et al.*, 1991; Zwemer *et al.*, 1992; Leavey *et al.*, 2004). NO<sub>2</sub> has a bleach-like odor, a reddish-brown to yellow color, and leaves a yellow stain on silage, wood, or other contact materials. It is heavier than air. The most commonly encountered source in veterinary medicine is from silos that have been recently filled with fresh organic material (notably corn or other grains) or in silage pits. NO<sub>2</sub> is formed when nitric oxide in fresh silage or silo contents comes in contact with oxygen in the air. Silage gas also typically contains carbon dioxide. Silo gas has been a cause of mortality in dairy cattle (Haynes, 1963; Verhoeff *et al.*, 2007).

Toxic (and potentially lethal) levels of NO<sub>2</sub> can develop within hours on top of the material in the silo or silage pit (Groves and Ellwood, 1989). Within silos, the gas tends to seep down through the silo chute. The risk is highest when feedstuffs that are high in nitrates are ensiled or used for silage. Gas production typically peaks at approximately 24h, but it may last for several days. Other veterinary-relevant sources of NO<sub>2</sub> include kerosene heaters, unvented gas stoves and gas heaters, and tobacco smoke.

### Toxic dose

The U.S. EPA National Ambient Air Quality Standards lists 0.053ppm as the 24-h time-weighted average upper limit for NO<sub>2</sub> in air. Levels of 25.9ppb (SD, 18.1ppb) are known to increase the risk of pediatric asthma in humans (Belanger *et al.*, 2006). Brief exposures to 200ppm can be fatal, and exposure to 50ppm can result in significant pulmonary edema. A 1-h exposure to 1ppm is associated with deleterious effects in the lung. The human odor threshold is approximately 0.1–0.2ppm.

### Toxicokinetics and toxicodynamics

NO<sub>2</sub> is relatively water insoluble, and accumulation in the upper respiratory tract is limited (Jonas, 1984; Douglas

*et al.*, 1989; Epler, 1989; Gurney *et al.*, 1991; Leavey *et al.*, 2004). The high levels of carbon dioxide present in silage gas may stimulate deeper inspiration of the gas, resulting in a higher delivered dose in the deep lung.

### **Pathophysiology**

NO<sub>2</sub> dissolves in water to produce nitrous and nitric acids, which are irritant and corrosive (Jonas, 1984; Douglas *et al.*, 1989; Epler, 1989; Gurney *et al.*, 1991; Leavey *et al.*, 2004). Free radical generation and associated damage are also an important part of the pathophysiology. The acids are also immunosuppressive and result in a reduced resistance to infection. With prolonged or high levels of exposure, NO<sub>2</sub> is absorbed. Absorbed NO<sub>2</sub> binds with high affinity to hemoglobin, forming nitrosyl hemoglobin, which is further oxidized to methemoglobin. Methemoglobinemia produces a left shift of the hemoglobin:oxygen disassociation curve, further impairing tissue oxygen delivery.

### **Vulnerable populations**

Individuals with pre-existing reactive airway disorders, asthma, and lung disease are likely to be at greater risk.

### **Clinical presentation**

Clinical disease is associated most with harvest season. The clinical presentation depends on the concentration and duration of exposure (Jonas, 1984; Pladson, 1984; Douglas *et al.*, 1989; Epler, 1989; Gurney *et al.*, 1991; do Pico, 1992; Zwemer *et al.*, 1992; Stepanek *et al.*, 1998; Leavey *et al.*, 2004). High levels can produce sudden death due to bronchiolar spasm, laryngeal spasm, reflex respiratory arrest, and/or asphyxia. Lower exposures may be asymptomatic, mild, and self-limiting or result in eye irritation, pulmonary edema, and/or acute respiratory distress syndromes. Mucous membrane irritation is uncommon because NO<sub>2</sub> does not tend to dissolve onto wet mucous membrane surfaces.

Failure to adequately treat significant NO<sub>2</sub> exposure with corticosteroids results in bronchiolitis obliterans, particularly in the small airways and alveolar ducts, and permanent restrictive lung disease. Bronchiolitis obliterans can develop weeks or months following the initial exposure.

### **Diagnostic testing**

Apart from air sampling/monitoring, there are no specific diagnostic tests.

### **Postmortem findings**

Chemical pneumonitis and pulmonary edema primarily located in the deep lung are the predominant findings.

Emphysema may be present, depending on the duration of the toxidrome. Typically, type I pneumocytes, pulmonary mast cells, and ciliated airway cells are the most affected cell types.

### **Treatment**

The immediate treatment priority is the prompt removal of the patient from the source of exposure. Rescuers need to take great care not to become casualties in such circumstances. The next treatment priority is the administration of 100% oxygen and resuscitation. Pulmonary edema may develop up to 48h following exposure, so volume expanders and fluid therapy must be used with great caution during this period. Corticosteroids are important for reducing the risk of bronchiolitis obliterans. The use of broad-spectrum antibiotics with the objective of preventing bronchopneumonia may be justified. Subsequent follow-up management of reactive airway syndromes and asthma may be required.

### **Prognosis**

The prognosis depends entirely on the degree of lung damage and whether or not bronchiolitis obliterans and restrictive lung disease develop (Jonas, 1984; Douglas *et al.*, 1989; Epler, 1989; Gurney *et al.*, 1991; Zwemer *et al.*, 1992; Leavey *et al.*, 2004). In many cases, the toxidrome is mild and self-limiting; however, it may take months for pulmonary function to return to normal. Permanent mild loss of pulmonary function is relatively common.

### **Prevention**

The following methods can prevent exposure: stay out of silos during the 2-week danger period after the initial filling; close all silo doors before filling; use outside ladders rather than internal ladders in silos; if the silo is not completely full, remove the doors that lead down to the silage; enter the silo only with a complete oxygen support system; ventilate the silo by opening the cover flaps and running the silo blower for 24–48h before entering; never enter the silo alone or without a lifeline for rescue during the danger period; use a personal air monitor; and install air monitors in areas where NO<sub>2</sub> is likely to concentrate.

## **Gaseous ammonia**

### **Overview, uses, and sources of exposure**

Within the veterinary context, ammonia (NH<sub>3</sub>) is most commonly encountered anywhere decaying organic matter is present, particularly urine and feces (Roney *et al.*, 2004). In this context, it is ubiquitous within intensive animal production facilities. Swine and poultry production facilities are notorious for containing toxic levels of

TABLE 59.3 Dose thresholds for gaseous ammonia

Concentration (ppm)	Effect
≤25	8-h time weighted average human exposure limit
25–50	Detectable odor; unlikely to experience adverse effects
50–100	Mild eye and upper respiratory tract irritation; may develop tolerance in 1 or 2 weeks with no adverse effects thereafter
140	Moderate eye irritation; no long-term sequelae in exposures of less than 2 h
400	Moderate throat irritation
500	Immediately dangerous to life and health (human)
700	Immediate eye injury
1000	Severe airway injury
1700	Immediate laryngospasm
2500–6500	Sloughing and necrosis of airway mucosa, chest pain, acute lung injury, and bronchospasm
5000	Rapidly fatal

Data from Roney *et al.* (2004).

ammonia that are often higher than acceptable human threshold limit values (Sigurdarson *et al.*, 2004; Davis and Morishita, 2005; McDonnell *et al.*, 2008). Ammonia is lighter than air and will thus tend to rise from manure pits (Roney *et al.*, 2004). Ammonia is still used as a refrigerant, and leaks in such systems remain a common cause of serious incidents. The burning of nylon, silk, wood, and melamine also results in considerable production of  $\text{NH}_3$ .  $\text{NH}_3$  is a major component of many common household cleaning and bleaching products, and the mixing of these products with those that contain chlorine results in the liberation of chloramines, which are highly irritant and potentially dangerous (Pascuzzi and Storrow, 1998).

High-pressure anhydrous ammonia gas is a commonly used fertilizer in some locations because it is often the cheapest source of nitrogen. Errors in handling and leakage represent a substantial risk (George *et al.*, 2000; Fitzgerald *et al.*, 2006; Welch, 2006).

Anhydrous ammonia is an important component in the manufacture of methamphetamines (Bloom *et al.*, 2008). Theft of anhydrous ammonia from farms has become commonplace and has resulted in numerous serious incidents (Amshel *et al.*, 2000; Latenser and Lucktong, 2000; Lessenger, 2004; Welch, 2006). Due to the unique chemical properties of anhydrous ammonia, it can exert extremely high pressures even at relatively low temperatures; thus, specialized containers and equipment are required for safe handling of the gas. Attempts at anhydrous ammonia theft often involve the use of propane tanks that are not designed to handle the physicochemical properties of

anhydrous ammonia, and the risk of explosion of these types of containers is high. Veterinarians and farmers should be extremely cautious around apparently empty propane tanks that have blue- or green-discolored valves or if the tanks have frost on them. Anhydrous ammonia may have been inappropriately stored in the container, and the container's brass, copper, or galvanized valve fittings may be compromised.

Liquid anhydrous ammonia expands to many times its original volume when released into air and forms large, highly dangerous vapor clouds. Aerosolized liquid anhydrous ammonia may behave as a dense gas and accumulate in low-lying spaces, even though it is normally lighter than air.

### Toxic dose

The concentration of  $\text{NH}_3$  in well-ventilated animal production facilities should remain below 30 ppm. The human odor threshold is approximately 10 ppm. The human dose response to  $\text{NH}_3$  is shown in Table 59.3. Adverse effects occur at concentrations greater than approximately 75 ppm. Anhydrous ammonia has an 8-h time-weighted average maximum acceptable exposure of 25 ppm, a short-term exposure limit of 35 ppm, and an immediately dangerous to life and health level of 500 ppm.

### Toxicokinetics and toxicodynamics

$\text{NH}_3$  is highly water soluble and thus tends to primarily affect the upper respiratory tract, mucous membranes, and the eye (Close *et al.*, 1980; O'Kane, 1983; Robinson *et al.*, 1990; Chao and Lo, 1996; Brautbar *et al.*, 2003; Makarovsky *et al.*, 2008). However, deeper structures may be affected if the upper respiratory tract "sink" is overwhelmed.

### Pathophysiology

$\text{NH}_3$  is irritant and/or corrosive depending on the concentration.  $\text{NH}_3$  reacts with tissue water to produce ammonium hydroxide, a strong alkali (Close *et al.*, 1980; Robinson *et al.*, 1990; Chao *et al.*, 1996; Brautbar *et al.*, 2003; Makarovsky *et al.*, 2008). The reaction is exothermic and capable of producing significant tissue burns. Ammonium hydroxide produces typical alkaline liquefaction necrosis. Alkali liquefaction necrosis results in deeper tissue damage than that caused by an equipotent acid. In addition, ammonium hydroxide tissue breakdown liberates water, aiding the further conversion of  $\text{NH}_3$  to ammonium hydroxide. Mild exposure primarily affects the upper respiratory tract and eyes, whereas more severe exposure may affect the entire respiratory tract. The degree of damage to the respiratory tract is dependent on the depth of inhalation, duration of exposure, concentration, and the pH change of the tissue fluids. Even mild levels of exposure can reduce pulmonary



clearance and potentially increase the risk of infections of the respiratory tract. Within animal facilities, ammonia is generally adsorbed by dust particles, which enable deeper penetration of the lung compared with gaseous  $\text{NH}_3$  (Kim *et al.*, 2008). In such circumstances, the toxicological thresholds for adverse effects may be lower than for gaseous  $\text{NH}_3$ .

Liquid anhydrous ammonia ( $-33^\circ\text{C}$ ) freezes tissue on contact and can produce deep, irreversible skin damage (Amshel *et al.*, 2000; Latenser and Lucktong, 2000; Lessenger, 2004; Welch, 2006).

### **Vulnerable populations**

Individuals with pre-existing lung disease, reactive airway syndromes, and/or asthma are at significantly greater risk.

### **Clinical presentation**

Clinical signs associated with relatively low-level exposures pertain to eye and upper respiratory tract irritation: shallow breathing, excessive lacrimation, nasal discharge, keratoconjunctivitis, corneal opacity, atrophic rhinitis, dyspnea, hemoptysis, hoarse voice, dysphagia, reduced production, and possibly increased rates of respiratory infections. Higher exposures can result in severe pulmonary disease and possibly acute respiratory distress.

### **Diagnostic testing**

Air monitoring is the most effective form of diagnostic testing.

### **Postmortem findings**

Irritation and possibly corrosion of the respiratory tract, particularly the upper tract, and exposed surfaces of the eye dominate the gross findings. Histological findings may include hyperplasia of the bronchiolar and alveolar epithelium.

### **Treatment**

The immediate treatment priority is the prompt removal of the patient from the source of exposure. Rescuers need to take great care not to become casualties in such circumstances. Copious skin and eye irrigation should be performed for at least 20 min (taking care to avoid hypothermia). Patients should then be treated for acute respiratory distress and/or burns. In animal production facilities, the best treatment is to improve ventilation and to reduce the accumulation of animal wastes within the facilities.

### **Prognosis**

The prognosis depends on the severity, depth, and chronicity of respiratory tract injury. Reactive airway diseases and

asthma are common following  $\text{NH}_3$  exposures. Chronic respiratory sequelae may occur with severe exposures.

### **Prevention**

Improved ventilation and removal of wastes is the most effective form of prevention within intensive animal facilities. The use of air monitors (personal and space) and personal protective equipment has reduced human casualties. Veterinarians and farmers should have a very high degree of suspicion regarding areas where high levels of  $\text{NH}_3$  might be present, particularly enclosed spaces. Improved security of liquid anhydrous ammonia storage facilities has been recommended.

## **Smoke inhalation**

### **Overview, uses, and sources of exposure**

Smoke inhalation injury results from a combination of exposure to gaseous combustion products, particulate matter (which may be superheated), and superheated air (Lee-Chiong, 1999; Alarie, 2002; Enkhbaatar and Traber, 2004; Fitzgerald *et al.*, 2006; Lee and Mellins, 2006; Schnepf, 2006; Sicoutris and Holmes, 2006; Stefanidou *et al.*, 2008). Thus, the syndrome results from a combination of thermal, gas, and particle effects. Smoke inhalation always involves some degree of carbon monoxide and cyanide poisoning. Combustion of plastics, polyurethane, wool, silk, nylon, nitriles, rubber, and paper leads to the production of cyanide gas. Other combustion products may include acrolein and other reactive aldehydes (organic combustion) and also chlorine, ammonia, ketones, hydrocarbons, and various acids (combustion of rubber and plastics).

### **Toxic dose**

The toxic dose depends on the source.

### **Pathophysiology**

Thermal damage primarily occurs in the upper respiratory tract, particularly in the oropharyngeal area, due to the poor heat conductivity of air and high dissipation of heat in the upper airways (Clark *et al.*, 1989, 1990; Clark, 1992; Lee-Chiong, 1999; Alarie, 2002; Enkhbaatar and Traber, 2004; Fitzgerald *et al.*, 2006; Lee and Mellins, 2006; Schnepf, 2006; Sicoutris and Holmes, 2006; Stefanidou *et al.*, 2008). Both pulmonary irritation and systemic effects occur. Systemic effects occur primarily due to direct and chemical asphyxiation. Chemical asphyxiation most commonly occurs as a result of a combination of carbon monoxide and cyanide poisoning. Methemoglobinemia occurs due to direct heat denaturation of hemoglobin, as well as the inhalation of nitrites.

### Vulnerable populations

Individuals with pre-existing lung disease, reactive airway syndromes, and/or asthma are at significantly greater risk.

### Clinical presentation

Any individual with facial burns is likely also to have concurrent smoke inhalation injury. Individuals who no longer have eyelashes are very likely to have concurrent ocular injury. Common early clinical signs of respiratory tract injury include hoarseness and a change in voice, carbonaceous nasal discharge or sputum, coughing, tachypnea, and use of accessory respiratory muscles (Clark *et al.*, 1989, 1990; Clark, 1992; Lee-Chiong, 1999; Alarie, 2002; Enkhbaatar and Traber, 2004; Fitzgerald and Flood, 2006; Lee and Millens, 2006; Schnepp, 2006; Sicoutris and Holmes, 2006; Stefanidou *et al.*, 2008). Later clinical signs are related to progressive pulmonary decline and acute respiratory distress.

### Diagnostic testing

Bronchoscopy is the definitive diagnostic procedure. Clinical chemistry may indicate metabolic acidosis resulting from a combination of hypoxia, CO poisoning, cyanide poisoning, and methemoglobinemia. Elevated serum lactate is a relatively sensitive indication of cyanide poisoning. Patients may have lowered blood cholinesterases because the pyrolysis products of many phosphorus-based fire retardants are anticholinesterases. Pulse oximetry may provide misleading information due to the presence of COHb and methemoglobinemia. If available, pulse co-oximetry is preferable.

Chest radiographs are commonly normal after smoke inhalation, and the diagnostic accuracy of the technique is low. Chest computerized tomography (CCT) is a preferable imaging technique. CCT abnormalities include ground-glass opacities in a peribronchial distribution and/or patchy peribronchial consolidations. Such abnormal CCT findings may be present within a few hours following smoke inhalation. Pulmonary function tests are sensitive indicators of smoke inhalation injury, but they are rarely readily available in veterinary practice.

### Treatment

The immediate treatment priority is the prompt removal of the patient from the source of exposure. Rescuers need to take great care not to become casualties in such circumstances. The next treatment priority is the administration of 100% oxygen, resuscitation, and maintenance of airway patency (Clark *et al.*, 1989, 1990; Clark, 1992).

Unfortunately, there is no specific treatment for the tissue damage and increased risk of infection associated

with smoke inhalation. Studies in humans have indicated that positive pressure ventilation with low tidal volumes (3–5 mL/kg) and positive end-expiratory pressure may increase short-term survival (Cancio, 2009). High-frequency percussive ventilation, whole-body hypothermia, and hyperbaric oxygen may also decrease mortality (Reper *et al.*, 1998; Thom *et al.*, 2001). Unfortunately, none of these techniques are readily available in veterinary medicine. Induction of methemoglobinemia for the treatment of cyanide poisoning may be dangerous if significant CO poisoning is also present. However, the use of hydroxocobalamin (Cyanokit) in combination with low-dose sodium thiosulfate as a cyanide antidote may be considered (Borron *et al.*, 2007a,b). Bronchodilator therapy is almost always indicated in cases of smoke inhalation.

Patients with significant smoke inhalation commonly require concurrent treatment for burns, a subject that is beyond the scope of this chapter.

### Prognosis

The prognosis is entirely dependent on the degree of smoke injury and associated injury. Significant smoke inhalation always has a guarded prognosis.

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# Alcohols and glycols

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## INTRODUCTION

Alcohols comprise a class of organic compounds composed of a hydrocarbon chain and a hydroxyl group. Alcohols that have one hydroxyl group are called monohydric, which include methanol, ethanol, and isopropanol, which are the three alcohols most commonly responsible for alcohol toxicosis. Alcohols are also classified as primary, secondary, or tertiary, according to the number of carbon atoms bonded to the carbon atom to which the hydroxyl group is bonded. Ethanol and methanol are primary alcohols, and isopropanol is a secondary alcohol. Glycols have two hydroxyl groups in their molecules and are dihydric. Ethylene glycol (EG) is the most common glycol responsible for poisonings, and EG poisoning is significantly more common in domestic animals than is alcohol toxicosis. Propylene glycol and butylene glycol are much less toxic than EG.

Alcohols and glycols are initially metabolized by hepatic alcohol dehydrogenase (ADH). Toxicosis from alcohols and glycols results in central nervous system (CNS) depression, ranging from decreased motor function to decreased consciousness, hypothermia, hypotension, coma and death from respiratory depression, and cardiovascular collapse. However, metabolites of the alcohols and glycols vary greatly in their toxicity. Metabolites of EG are nephrotoxic and result in acute renal failure. In primates, metabolites of methanol may result in blindness and permanent neurologic abnormalities.

## BACKGROUND

Reports of alcohol toxicosis are relatively quite rare in domestic animals compared to the reported incidence

in human beings. Ethanol, methanol, and isopropanol poisoning are all quite common in humans. Methanol and isopropanol are usually ingested by adults as a substitute for ethanol or in an attempt to commit suicide. In children, ingestion is usually accidental. Propylene glycol toxicosis is relatively rare in both humans and domestic animals. Butylene glycol toxicosis is also rare, but the incidence in humans has increased recently as it is regarded as a neuromodulatory “recreational” drug (Irwin, 1996). EG toxicosis is relatively common, both in humans and in domestic animals (Barton and Oehme, 1981; Mueller, 1982; Rowland, 1987; Hornfeldt and Murphy, 1998). In humans, it is ingested either accidentally, as a substitute for ethanol, or to commit suicide; approximately 5000 episodes are reported in the United States each year (Litovitz *et al.*, 1997). The vast majority of these poisonings are unintentional, and approximately one-third of the cases occur in children (Litovitz *et al.*, 1997). EG is the most common cause of human poisoning in some countries, such as Poland (Sienkiewicz and Kwiecinski, 1992).

Most incidents of EG toxicosis in domestic animals are also accidental, although malicious poisonings also occur. The mortality rate in dogs is reported to range from 50 to 70% (Barton and Oehme, 1981; Rowland, 1987; Connally *et al.*, 1996), and it is likely even higher in cats. EG intoxication is the second most common cause of fatal poisoning in animals, according to the American Association of Poison Control Centers (Hornfeldt and Murphy, 1998).

The first reported case of EG intoxication in a human occurred in 1930 (Anonymous, 1930), but the toxicity of EG was not fully realized until 1938 when 76 people died after consuming an elixir of sulfanilamide containing 96% diethylene glycol (Geiling and Cannon, 1938). Since then, many reports of EG poisoning in humans and animals have been published.

## ALCOHOL TOXICOSES

### Ethanol toxicosis

Ethanol (ethyl alcohol) has the structural formula  $C_2H_5OH$ , a molecular weight of 46 Da, and is the alcohol in alcoholic beverages. The percentage of ethanol in alcoholic beverages is one-half of the drink's proof value. It is also used in perfumes and mouthwashes. Ethanol toxicosis in dogs has been associated with ingestion of bread dough (Thrall *et al.*, 1984a; Suter, 1992; Means, 2003). Uncooked bread dough contains *Saccharomyces cerevisiae* (common brewer's and baker's yeast), which metabolizes carbohydrate substrates to ethanol and carbon dioxide. Ethanol poisoning in dogs has also been associated with rotten apples (Kammerer *et al.*, 2001) and alcoholic beverages (van Wuijckhuise and Cremers, 2003). Ethanol toxicosis can also occur in dogs and cats when ethanol is given intravenously (i.v.) as a competitive substrate to treat EG toxicosis. In a study in which cats were experimentally poisoned with EG and then treated with intraperitoneal (i.p.) ethanol at a dose of 5 mL of 20% ethanol/kg body weight every 6 h, serum ethanol concentrations ranged from as low as 16 mg/dL at 6 h post-i.p. ethanol to as high as 240 mg/dL 30 min post-i.p. ethanol. Cats with serum ethanol concentrations of more than 200 mg/dL appeared to be near respiratory arrest and were hypothermic (Thrall *et al.*, 1988).

### Toxicokinetics

Ethanol is rapidly absorbed from the gastrointestinal (GI) tract; the rate of absorption can be slowed by the presence of food in the stomach or small intestine. Clinical findings in dogs and cats with ethanol intoxication can be correlated with blood ethanol concentration (BEC). Clinical signs include ataxia, lethargy, sedation, hypothermia, and metabolic acidosis. Time of onset of clinical signs is dependent on the dose ingested and the amount of food present in the GI tract, but it usually occurs within an hour of ingestion. Although respiratory depression usually develops when the concentration is between 400 and 500 mg/dL, death has been reported in a human being with BEC as low as 260 mg/dL (Maling, 1970).

### Mechanism of action

The mechanism of action of alcohol on the CNS is related in part to its interactions with biomembranes and its probable inhibition of gamma-aminobutyric acid receptors (Valentine, 1990).

### Diagnosis and treatment

A diagnosis can be made based on history, clinical signs, increased plasma osmolality and osmole gap, metabolic

acidosis, and BEC, which can be measured by most laboratories. Hyperosmolality as a result of alcohol toxicosis must be determined using a freezing-point depression osmometer because vapor pressure osmometers do not detect the osmotic effects of alcohols (Champion *et al.*, 1975). It is important to differentiate EG toxicosis from alcohol toxicosis. The clinical signs and early laboratory findings can be similar. However, patients with EG toxicosis must be given ADH inhibitors such as 4-methylpyrazole to prevent the formation of toxic metabolites, whereas patients with ethanol toxicosis can be treated supportively. Severe ethanol intoxication usually requires mechanical ventilation using a cuffed endotracheal tube. Hypothermia may develop, and alterations in hydration, electrolyte, and acid-base status should be corrected (Richardson, 2006).

### Methanol toxicosis

Methanol (methyl alcohol and wood alcohol) has the molecular structure  $CH_3OH$ ; a molecular weight of 32 Da; and is widely used as a solvent, fuel (Sterno), gasoline additive, antifreeze, and windshield washer fluid (30–40% methanol). The minimum lethal dose in dogs is between 5.0 and 11.25 mL/kg, and in human beings it is 1.25 mL/kg (Valentine, 1990). Methanol toxicosis is rare in dogs, but it has been reported in a dog that chewed open a bottle of 98% methanol antifreeze (Hurd-Kuenzi, 1983).

### Toxicokinetics

Ingested methanol is absorbed quickly from the GI tract, and peak methanol concentrations occur within 30–60 min following ingestion (Barceloux *et al.*, 2002). Toxicosis has also been reported following inhalation or dermal absorption. Methanol is much more toxic to human beings and nonhuman primates than it is to other mammals. Methanol is metabolized by ADH to formaldehyde, which is oxidized to formic acid by formaldehyde dehydrogenase. In mammals other than primates, formic acid is metabolized relatively rapidly to carbon dioxide and water. Formic acid is metabolized less efficiently in primates.

### Mechanism of action

Formic acid is responsible for ocular and CNS lesions in primates as a result of inhibition of cytochrome oxidase (Roe, 1982). Blindness and permanent neurological abnormalities are common sequel in primates.

### Diagnosis and treatment

Clinical signs in animals other than primates are similar to those seen with ethanol toxicosis and are primarily

related to CNS depression and metabolic acidosis. Vomiting and abdominal pain may be seen. In primates, following the initial nausea and CNS depression, a latent period of approximately 12–24 h is followed by metabolic acidosis and impaired visual function. Laboratory findings in primates include hyperosmolality, increased anion gap, and severe metabolic acidosis (Bischoff, 2006a). Diagnosis can be made by history and measurement of blood methanol concentrations (or formic acid in primates).

Treatment in nonprimates is symptomatic and similar to treatment for ethanol toxicosis. Primates are treated with 4-methylpyrazole to compete with ADH and inhibit metabolism (Barceloux *et al.*, 2002) or, alternatively, with ethanol. Hemodialysis is also used to remove formic acid. Folic acid is given i.v. to enhance formic acid metabolism. It is probably inappropriate to treat methanol toxicosis in nonprimates with ethanol because ethanol contributes to the sedation, and the metabolites of methanol do not cause blindness in dogs, as they do in primates.

### Isopropanol toxicosis

Isopropanol (isopropyl alcohol) has the structural formula  $\text{CH}_3\text{CH}(\text{OH})\text{CH}_3$ ; a molecular weight of 60 Da; and is found in rubbing alcohol (70%), antifreeze, detergents, window cleaning products, and disinfectants. Ingestion is the usual cause of poisoning in humans, although toxicity from inhalation and topical absorption has been reported. Isopropanol toxicosis is rare in domestic animals, possibly due to its bitter taste. It has been reported in a horse that was mistakenly administered isopropanol via nasogastric intubation for colic; the isopropanol was mistaken for mineral oil (Somerville and Plumlee, 1996).

#### Toxicokinetics

Isopropanol is approximately twofold more toxic than ethanol. It is rapidly absorbed from the GI tract, and approximately 80% is metabolized to acetone, which is also a CNS depressant, but acetone has a much longer half-life (16–20 h) than does alcohol.

#### Diagnosis and treatment

Clinical signs associated with isopropanol toxicosis are similar to those for ethanol toxicosis and include CNS depression, hypotension, vomiting, and abdominal pain due to severe gastritis, which is secondary to direct irritation. The breath of patients who have ingested isopropanol has a characteristic acetone-like odor (Somerville and Plumlee, 1996). Treatment is supportive and includes fluids, correction of acid–base abnormalities, and assisted respiration if necessary (Oehme and Kore,

2006). Hemodialysis is effective in removing isopropanol and acetone, and 4-methylpyrazole will prevent the metabolism of isopropanol to acetone.

## GLYCOL TOXICOSES

### Propylene glycol toxicosis

Propylene glycol (1,2-propanediol) has the structural formula  $\text{C}_3\text{H}_8\text{O}_2$  and a molecular weight of 76 Da. It is one of the least toxic of the glycols, and it is used as automotive antifreeze, an industrial and pharmaceutical solvent, in cosmetics, and a processed food additive for human and animal consumption. Although it is considered non-toxic compared to EG, it causes CNS depression and lactic acidosis when ingested in large quantities. When used as an additive in semi-moist cat food, it historically caused Heinz body formation in erythrocytes but did not cause an anemia when ingested in small quantities (Christopher *et al.*, 1989a,b; Weiss *et al.*, 1990; Bauer *et al.*, 1992a,b). However, cats eating such diets were more susceptible to other additional causes of oxidative injury, and although overt anemia may not occur, red cells with Heinz bodies have a reduced life span. Consequently, it is no longer used as an additive in cat foods.

The oral median lethal dose for propylene glycol in dogs has been reported to be as low as 9 mL/kg (Bischoff, 2006b). Fatal cases of malicious propylene glycol toxicosis have been reported in dogs (Bischoff, 2006b). It is considered relatively unpalatable to dogs (Marshall and Doty, 1990). Fatal propylene glycol toxicosis was reported in a horse that was inadvertently given 7.6 mL/kg propylene glycol orally instead of mineral oil for potential grain overload (Dorman and Hascheck, 1991). The horse had a serum concentration of 900 mg/dL propylene glycol at the time of death, 28 h following ingestion. Cause of death was presumably respiratory arrest. Propylene glycol toxicosis has been reported in at least two other horses with colic in which propylene glycol was mistaken for mineral oil (Myers and Usenik, 1969; McClanahan *et al.*, 1998); both of these horses survived. In both cases, the horses were given approximately 6 mL/kg body weight via nasogastric tube. Heinz body formation also occurs in horses ingesting propylene glycol (McClanahan *et al.*, 1998). Propylene glycol may be used to treat and prevent bovine ketosis, which may partially explain the availability and apparent ease with which it is confused with mineral oil.

#### Toxicokinetics

Propylene glycol is rapidly absorbed in the GI tract and oxidized by ADH to D and L isomers of lactic acid,



resulting in lactic acidosis. Almost all propylene glycol is metabolized within 24 h of ingestion.

### Diagnosis and treatment

Clinical signs depend on the quantity ingested and may include depression, ataxia, muscle fasciculations, hypotension, osmotic diuresis, respiratory arrest, and circulatory collapse. Clinical signs in ruminants are similar to those seen in other species and include ataxia, depression, and recumbency (Pintchuck *et al.*, 1993). Laboratory findings include metabolic acidosis, increased anion gap, and hyperosmolality of the plasma and the presence of Heinz bodies in cats and horses. Diagnosis is usually based on history of exposure and can be confirmed by measuring propylene glycol concentrations in urine and serum by gas chromatography. Treatment for all species is supportive and includes correction of hydration and acid-base abnormalities.

### Butylene glycol toxicosis

Butylene glycol (1,2-, 1,3-, and 1,4-butanediol) has the structural formula  $C_4H_{10}O_2$  and a molecular weight of 90 Da. Butylene glycol is used as antifreeze, as an industrial cleaner, and in cosmetics. It is also a component of polyurethane and is used to make Spandex. Although no published reports of butylene glycol toxicosis in domestic animals were found in the literature, there are numerous reports of human intoxications from butylene glycol or the metabolite gamma-hydroxybutyrate (Dyer, 1991; Mack, 1993). Butylene glycol and the metabolite gamma-hydroxybutyrate are used as "recreational" drugs and were once marketed by health food stores as a food additive for body-builders and to treat depression and insomnia.

1,3-Butanediol has been used as an antidote for experimental EG toxicosis in dogs because it is a competitive substrate for ADH (Thrall *et al.*, 1982; Murphy *et al.*, 1984; Cox *et al.*, 1992). Although it was found to be a more effective antidote than ethanol, in that more unmetabolized EG was excreted in the urine in patients treated with 1,3-butanediol, CNS depression was as severe or more severe than that induced by ethanol therapy, and plasma hyperosmolality and metabolic acidosis were more severe than with ethanol therapy (Thrall *et al.*, 1982).

### Toxicokinetics

Butylene glycol is metabolized by ADH to acetoacetate and gamma-hydroxybutyrate, the so-called "date rape" drug.

### Mechanism of action

Butylene glycol is a CNS depressant much like ethanol, due to the effect of the gamma-hydroxybutyrate on the

CNS, and in large quantities can result in seizures and respiratory arrest.

### Treatment

Therapy for butylene glycol toxicosis in humans is supportive, similar to therapy for ethanol toxicosis.

### Ethylene glycol toxicosis

EG has the structural formula  $C_2H_6O_2$  and a molecular weight of 62 Da. EG is used primarily as an antifreeze and windshield de-icing agent. Its small molecular weight makes it very effective in lowering the freezing point of water. EG is also used as a cryoprotectant for embryo preservation; in the manufacture of polyester compounds; as a solvent in the paint and plastic industries; and as an ingredient in photographic developing solutions, hydraulic brake fluid and motor oil, and inks and wood stains (Davis *et al.*, 1997). The most readily available source of EG in the home is antifreeze solutions, which consist of approximately 95% EG.

### Toxicokinetics

Unlike the other alcohols (with the exception of methanol in primates) and glycols, the metabolites of EG are very toxic. EG is initially oxidized to glycoaldehyde by ADH, and glycoaldehyde is then oxidized to glycolic acid and then to glyoxylic acid. Glyoxylic acid is primarily converted to oxalic acid but may follow several metabolic pathways; end products may also include glycine, formic acid, hippuric acid, oxalomalic acid, and benzoic acid. Calcium is bound to oxalic acid, resulting in calcium oxalate crystal formation. Calcium oxalate crystal deposition is widespread but is most severe in the kidney, and crystalluria is a consistent finding in animals producing urine (Grauer *et al.*, 1984; Thrall *et al.*, 1984b).

### Mechanism of action

EG per se has no major effects other than GI irritation and increased serum osmolality. Glycoaldehyde, the first metabolite, is thought to be primarily responsible for CNS dysfunction; respiration, glucose, and serotonin metabolism are depressed; and CNS amine concentrations are altered (Parry and Wallach, 1974; Gordon and Hunter, 1982). Hypocalcemia secondary to calcium oxalate deposition may contribute to CNS signs, although the concurrent metabolic acidosis shifts calcium to the ionized active state, reducing the chances of hypocalcemia-associated clinical signs. Acidosis is also thought to lead to altered levels of consciousness and cerebral damage. Most of the metabolites are very cytotoxic to renal tubular epithelium, and some

renal epithelial and interstitial damage may be associated with calcium oxalate crystal formation within the renal tubules (de Water *et al.*, 1999). Renal epithelial cell death appears to be due primarily to destruction of cytoplasmic organelles, especially mitochondria (Bachman and Goldberg, 1971). Metabolic acidosis is often severe and has a deleterious effect on multiple organ systems. Glycolic acid accumulation is the primary cause of the metabolic acidosis associated with EG intoxication (Jacobsen *et al.*, 1984), although other acid metabolites also contribute. Glycolic acid accumulates because the lactic dehydrogenase enzyme that metabolizes glycolic to glyoxylic acid becomes saturated.

### Toxicity

Before it is metabolized, EG is no more toxic than ethanol, although EG is a more potent CNS depressant than ethanol (Berger and Ayyar, 1981). However, EG is biotransformed to highly toxic metabolites that result in severe metabolic acidosis and acute renal failure, hallmarks of EG poisoning (Thrall *et al.*, 1984b; Dial *et al.*, 1994a,b; Davis *et al.*, 1997). The minimum lethal dose of undiluted EG is 6.6 mL/kg in the dog (Kersting and Nielson, 1966) and 1.5 mL/kg in the cat (Milles, 1946).

### Clinical signs

Clinical signs are dose dependent and can be divided into those caused by unmetabolized EG and those caused by its toxic metabolites. The onset of clinical signs is almost always acute. Early clinical signs are usually observed 30 min after ingestion and often last until approximately 12 h after ingestion; they are primarily associated with EG-induced gastric irritation and high EG blood concentrations. These signs commonly include nausea and vomiting, CNS depression, ataxia and knuckling, muscle fasciculations, decreased withdrawal reflexes and righting ability, hypothermia, and osmotic diuresis with resultant polyuria and polydipsia (Grauer *et al.*, 1984; Thrall *et al.*, 1984b; Connally *et al.*, 1996). As CNS depression increases in severity, dogs drink less but osmotic diuresis persists, resulting in dehydration. In dogs, CNS signs abate after approximately 12 h, and patients may briefly appear to have recovered. Cats usually remain markedly depressed and do not exhibit polydipsia. Animals may be severely hypothermic, particularly if housed outside during the winter months. Clinical signs associated with the toxic metabolites are primarily related to oliguric renal failure, which is evident by 36–72 h following ingestion in dogs and by 12–24 h following ingestion in cats. Clinical signs may include severe lethargy or coma, seizures, anorexia, vomiting, oral ulcers and salivation, and oliguria with isosthenuria. Anuria often develops 72–96 h after ingestion. The kidneys are often swollen and painful, particularly in cats.

### Early laboratory abnormalities

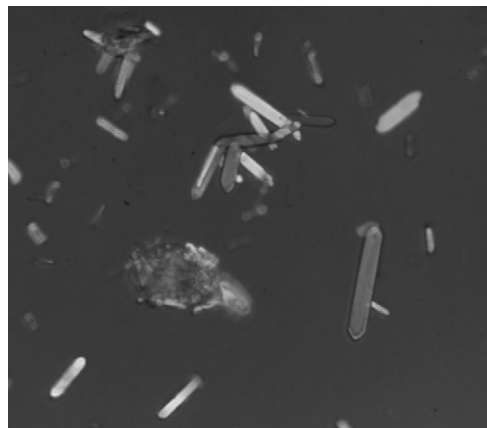
Abnormal laboratory findings can also be divided into those associated with early EG intoxication, which may be related to the presence of EG *per se* or to its toxic metabolites, and those associated with late EG intoxication, most of which are related to renal failure. Early abnormalities are primarily due to the presence of acid metabolites of EG in the serum that result in metabolic acidosis and include decreased plasma bicarbonate concentration and increased anion gap. In addition, hyperphosphatemia may occur due to ingestion of a phosphate rust inhibitor present in some commercial antifreeze products (Grauer *et al.*, 1984; Connally *et al.*, 1996). The decreased plasma bicarbonate ( $\text{HCO}_3^-$ ) concentration can be seen as early as 1 h following EG ingestion. Metabolites of EG significantly increase the pool of unmeasured anions and cause an increased anion gap. The anion gap is increased by 3 h after ingestion, peaks at 6 h after EG concentration, which peaks 1–6 h following ingestion, and EG is usually no longer detectable in the serum or urine 48–72 h after ingestion (Thrall *et al.*, 1982; Grauer *et al.*, 1984; Dial *et al.*, 1994a,b). Kits (e.g., Ethylene Glycol Test Kit, PRN Pharmacol, Pensacola, FL) are available that accurately estimate blood EG concentrations with a minimum detection limit of 50 mg/dL, and the results correlate well with other established methods of measuring EG concentrations such as gas chromatography (Dasgupta *et al.*, 1995), although the presence of propylene glycol or glycerol in the blood may cause a false-positive test reaction. Ethanol and methanol do not result in a false-positive test result. Cats may be intoxicated with a lethal dose of EG that is below the 50 mg/dL detectable level of the EG test kit. Therefore, if the test kit is negative and historical findings as well as clinical signs are compatible with EG ingestion, the recommendation is to initiate appropriate therapy for EG intoxication as well as submit a serum sample to a reference laboratory capable of determining a quantitative concentration.

Determination of serum osmolality is also useful for diagnosing early EG toxicosis, although other osmotically active, low-molecular-weight alcohols and glycols also increase serum osmolality (Ammar and Heckerling, 1996). Serum osmolality is increased by 1 h after ingestion of EG, increasing in parallel with serum EG concentrations (Dial *et al.*, 1994a,b). When measured serum osmolality (by osmometry) is compared to calculated serum osmolality, the difference is referred to as the osmole or osmolal gap. If calculated osmolality is not provided on the biochemical profile printout, osmolality in mOsm/kg may be calculated using the following formula:

$$1.86 (\text{Na}^+ + \text{K}^+) + \text{glucose}/18 + \text{BUN}/2.8 + 9$$

Normal serum osmolality is 280–310 mOsm/kg, and the normal osmole gap is less than 10 mOsm/kg. Serum osmolality as high as 450 mOsm/kg and an osmole gap as high as 150 mOsm/kg may be seen 3 h after ingestion, depending on the quantity of antifreeze ingested (Jacobsen *et al.*, 1982b; Grauer *et al.*, 1984). Both the gap and the measured osmolality may remain significantly high for approximately 18 h after ingestion. Multiplication of the osmole gap by 5 yields an approximate serum EG concentration in mg/dL (Burkhart and Kulig, 1990). Each 100 mg/dL increment increase in EG concentration contributes approximately 16 mOsm/kg H<sub>2</sub>O to the serum osmolality (Eder *et al.*, 1998). Simultaneous or sequential increases in osmole and anion gaps are very suggestive of EG intoxication. As EG is metabolized, its contribution to the osmole gap diminishes because the accumulating negatively charged metabolites do not contribute to the osmole gap (Eder *et al.*, 1998). Two types of instruments are used to measure osmolality – freezing point osmometers and vapor pressure osmometers. Because EG is nonvolatile (boiling point, 197°C), it is detected by either the freezing point or vapor pressure methods. However, methanol, ethanol, and other volatile compounds, although contributing to serum osmolality, may go undetected if assayed by the vapor pressure method. Most clinical laboratories use the freezing point method (Kruse and Cadnapaphornchai, 1994). Osmolality can be measured using serum or plasma; if the latter is used, heparin is the preferred anticoagulant. Other anticoagulants, such as EDTA, can markedly increase osmolality and can result in spurious increases in the osmole gap (Kruse and Cadnapaphornchai, 1994).

Dogs are isosthenuric (urine specific gravity of 1.008–1.012) by 3 h following ingestion of EG due to osmotic diuresis and serum hyperosmolality-induced polydipsia (Grauer *et al.*, 1984; Dial *et al.*, 1994a). The urine specific gravity in cats is also decreased by 3 h after ingestion but may be above the isosthenuric range (Dial *et al.*, 1994b; Fogazzi, 1996). Calcium oxalate crystalluria is a common finding and may be observed as early as 3 and 6 h after ingestion in the cat and dog, respectively, as a result of oxalic acid combining with calcium (Dial *et al.*, 1994a,b). Calcium oxalate monohydrate crystals are variably sized, clear, six-sided prisms (Figure 60.1) (Scully *et al.*, 1979; Godolphin *et al.*, 1980; Terlinsky *et al.*, 1981; Jacobsen *et al.*, 1982a; Kramer *et al.*, 1984; Foit *et al.*, 1985; Thrall *et al.*, 1985; Steinhart, 1990). In animals and people poisoned with EG, the monohydrate form is observed more frequently than the dihydrate form, which appears as an envelope or Maltese cross (Connally *et al.*, 1996; Eder *et al.*, 1998). Dumbbell or sheaf-shaped crystals are observed infrequently. The detection of calcium oxalate crystalluria, particularly the monohydrate form, provides strong supporting evidence for the diagnosis of EG poisoning (Fogazzi, 1996). Urinary pH consistently decreases following EG ingestion.



**FIGURE 60.1** Calcium oxalate monohydrate crystals (polarized light) from a dog with EG toxicosis.

Another diagnostic procedure that may be helpful in detecting early EG intoxication is examination of the oral cavity, face, paws, vomitus, and urine with a Wood's lamp to determine whether they appear fluorescent. Many antifreeze solutions manufactured today contain sodium fluorescein, a fluorescent dye that aids in the detection of leaks in vehicle coolant systems. The dye is excreted in the urine for up to 6 h following ingestion of the antifreeze (Winter *et al.*, 1990). A negative test does not eliminate the possibility of EG ingestion because not all antifreeze solutions contain the dye.

#### *Late laboratory abnormalities*

With the onset of renal damage and subsequent decreased glomerular filtration, serum creatinine and blood urea nitrogen (BUN) concentrations increase. In the dog, these increases begin to occur between 24 and 48 h following EG ingestion. In the cat, BUN and creatinine begin to increase approximately 12 h after ingestion; however, because cats do not develop polydipsia, this may be in part due to dehydration. Serum phosphorus concentrations increase at this time due to decreased glomerular filtration. Hyperkalemia develops with the onset of oliguria and anuria. Serum calcium concentration is decreased in approximately half of patients (Thrall *et al.*, 1984b; Connally *et al.*, 1996) and is due to formation of insoluble calcium oxalate. Clinical signs of hypocalcemia are infrequently observed because acidosis results in a shift to the ionized, physiologically active form of calcium. Serum glucose concentration is increased in approximately 50% of dogs and cats (Thrall *et al.*, 1984b; Connally *et al.*, 1996) and is attributed to inhibition of glucose metabolism by aldehydes, increased epinephrine and endogenous corticosteroids, and uremia. Animals presenting with late EG poisoning are likely to have little or no osmole gap increase but will have an increased osmolality (whether calculated or



measured) because of the azotemia and hyperglycemia. Animals remain isosthenuric in the later stages of toxicosis due to renal dysfunction and impaired ability to concentrate urine. Calcium oxalate crystalluria persists for as long as animals are producing urine. Urine abnormalities associated with renal damage may include hematuria, proteinuria, and glucosuria. Granular and cellular casts, white blood cells, red blood cells, and renal epithelial cells may be observed in the sediment of some patients (Thrall *et al.*, 1984b; Connally *et al.*, 1996).

### Treatment

Therapy for EG poisoning is aimed at preventing absorption, increasing excretion, and preventing metabolism of EG. Supportive care to correct fluid, acid-base, and electrolyte imbalances is also helpful. Although therapeutic recommendations have traditionally included induction of vomiting, gastric lavage, and administration of activated charcoal (Thrall *et al.*, 1995, 1998), it is likely that these procedures are not beneficial because of the rapidity with which EG is absorbed (Davis *et al.*, 1997). The most critical aspect of therapy is based on prevention of EG oxidation by ADH, the enzyme responsible for the initial reaction in the EG metabolic pathway (Parry and Wallach, 1974). Typically, dogs must be treated within 8 h following ingestion and cats must be treated within 3 h for treatment to be successful (Dial *et al.*, 1994a,b). However, this is somewhat dependent on the amount of EG ingested. Historically, treating EG toxicosis has been directed toward inhibiting EG metabolism with ethanol, a competitive substrate that has a higher affinity for ADH than EG (Penumarthy and Oehme, 1975; Bostrom and Li, 1980). Ethanol has numerous disadvantages because it enhances many of the metabolic effects of EG. Both ethanol and EG are CNS depressants, and it is the compounded CNS depression that most limits the usefulness of ethanol as an antidote. Additional disadvantages of ethanol treatment include its metabolism to acetaldehyde, which impairs glucose metabolism and is a cerebral irritant. Ethanol also contributes to metabolic acidosis by enhancing the formation of lactic acid from pyruvate and may potentiate hypocalcemia (Money *et al.*, 1989). Moreover, ethanol compounds the effects of EG-induced osmotic diuresis and serum hyperosmolality (Kruse and Cadnapaphornchai, 1994).

4-Methylpyrazole (fomepizole) has become the preferred antidote in dogs (Grauer *et al.*, 1987; Dial *et al.*, 1989; Connally *et al.*, 1996) and cats (Thrall *et al.*, 2006; Connally *et al.*, 2010). Fomepizole is an ADH inhibitor, not a competitive substrate, and it does not induce CNS depression (in dogs), diuresis, or hyperosmolality at the recommended dosage. The recommended dose of fomepizole for dogs is 20 mg/kg body weight i.v. initially, followed by 15 mg/kg i.v. at 12 and 24 h and 5 mg/kg i.v. at 36 h (Grauer *et al.*,

1987; Connally *et al.*, 1996; Thrall *et al.*, 2006). Cats must be given a much higher dose of fomepizole than dogs because feline ADH is less effectively inhibited by fomepizole than is canine ADH (Connally *et al.*, 2000, 2010). Cats are initially treated with 125 mg/kg fomepizole i.v. followed by 31.25 mg/kg i.v. fomepizole at 12, 24, and 36 h. The only adverse clinical sign that the authors have observed is CNS depression that appears to be fomepizole related (Connally *et al.*, 2010). If ingestion of a large dose of EG is suspected, repeating serum quantification tests can be performed to determine whether continuation of therapy beyond 36 h is necessary. Alternatively, additional doses of fomepizole can be administered empirically. Fomepizole is commercially available as Antizol-Vet (Orphan Medical, Minnetonka, MN), which can be conveniently reconstituted. Appropriate therapy also consists of i.v. fluids to correct dehydration, increase tissue perfusion, and promote diuresis. The fluid volume administered should be based on the maintenance, deficit, and continuing loss needs of the patient. Frequent measurement of urine production, serum urea nitrogen and creatinine, and blood pH, bicarbonate, ionized calcium, and electrolytes daily or twice daily will help guide fluid and electrolyte therapy (Grauer, 1998). Bicarbonate should be given slowly i.v. to correct the metabolic acidosis. Hypothermia can be controlled with blankets or the use of a pad with circulating warm water.

In animals that are azotemic and in oliguric renal failure on presentation, almost all of the EG has been metabolized, and treatment to inhibit ADH is likely to be of little benefit. However, ADH inhibitors should be given up to 36 h following ingestion to prevent the metabolism of any residual EG. Fluid, electrolyte, and acid-base disorders should be corrected and diuresis established, if possible. Diuretics, particularly mannitol, may be helpful. The tubular damage caused by EG may be reversible, but tubular repair can take weeks to months. Animals may take up to 1 year following EG toxicosis to regain concentrating ability, and some remain isosthenuric. Supportive care to maintain the patient during the period of renal tubular regeneration is necessary, and peritoneal dialysis may be useful (Shahar and Holmberg, 1985; Fox *et al.*, 1987; Crisp *et al.*, 1989). Hemodialysis has been attempted in dogs with EG-induced renal failure (DiBartola *et al.*, 1985) and has been shown to have a relatively good success rate in cats with acute renal failure (Langston *et al.*, 1997). Renal transplantation has also been used with variable success in cats with renal failure (Gregory *et al.*, 1992; Mathews and Gregory, 1997) and has been described in dogs (Nemeth *et al.*, 1997).

### Prognosis

EG has a very high potential for a lethal outcome, but with early recognition of the syndrome and timely institution of therapy, animals can be saved. The quantity of



EG ingested, rate of absorption, and time interval prior to institution of therapy are variables that affect the prognosis. The prognosis is excellent in dogs treated with fomepizole within 5h of ingesting EG. In a retrospective study of dogs with confirmed EG poisoning, all of the dogs that were azotemic when initially treated died. Of the dogs that did not have azotemia when initially treated, approximately 90% survived (Connally *et al.*, 1996). The prognosis for cats is reasonably good if treatment is instituted within 3h following ingestion (Dial *et al.*, 1994b). In contrast, the prognosis in humans who survive the initial syndrome of severe acidosis is very good. Terminal renal failure in humans is rare, and most human patients regain renal function by 2 months following EG poisoning (Davis *et al.*, 1997) likely due to the effectiveness of hemodialysis therapy in humans (Christiansson *et al.*, 1995).

## CONCLUSIONS

Ethanol, methanol, isopropanol, propylene glycol, butylene glycol, and marijuana toxicosis can produce ataxia and other CNS signs similar to those seen in acute EG poisoning but are much less common than EG toxicosis (Godbold *et al.*, 1979; Hurd-Kuenzi, 1983; Thrall *et al.*, 1984a; Suter, 1992). These disorders can be differentiated by the diagnostic laboratory tests discussed previously. Other causes of an increased anion gap include diabetic ketoacidosis and lactic acidosis; these disorders can also be differentiated by appropriate laboratory tests. Other causes of increased osmolality include ethanol, isopropanol, methanol, and propylene glycol toxicosis. Ethanol, like EG, can also produce hypocalcemia (Money *et al.*, 1989). Other differentials for acute renal failure include leptospirosis, ibuprofen and other nonsteroidal anti-inflammatory drug toxicosis, aminoglycoside antibiotics, hemolyticuremic syndrome, cholecalciferol toxicosis, grape and raisin toxicosis in dogs, and ingestion of oxalate-containing plants such as philodendron and lily toxicosis in cats (Brown *et al.*, 1985, 1996; Spyridakis *et al.*, 1986; Gunther *et al.*, 1988; Peterson *et al.*, 1991; Holloway *et al.*, 1993; Rivers *et al.*, 1996; Vaden *et al.*, 1997a,b; Poortinga and Hungerford, 1998; Forrester and Troy, 1999; Adin and Cowgill, 2000; Hovda, 2000; Rumbelha *et al.*, 2000; Singleton, 2001; Langston, 2002; Tefft, 2004). The majority of dogs with grape and raisin toxicosis are hypercalcemic, as are animals with cholecalciferol toxicosis (Fooshee and Forrester, 1990; Gwaltney-Brant *et al.*, 2001); hypercalcemia is not associated with EG toxicosis (Thrall *et al.*, 1984b; Connally *et al.*, 1996). Acute renal failure must be differentiated from acutely decompensated chronic renal failure. Carbamylated hemoglobin concentration

has been shown to be useful in making this differentiation (Vaden *et al.*, 1997b; Heiene *et al.*, 2001). In addition, animals with chronic renal failure may be anemic and in poor body condition. A history of the duration of clinical signs is also helpful. Continuing to increase the awareness of the toxicity of EG, as well as other alcohols and glycols, will aid in preventing exposure and result in earlier presentation of animals.

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# Petroleum

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## INTRODUCTION

This chapter primarily discusses the toxicology of crude petroleum and pollution generated during the production of petroleum. Emphasis is placed on domestic animals. Domestic and wild animals can also be exposed to environmental petroleum. Crude petroleum can be released into the environment during well blowouts, leaks at wellheads, pipeline leaks, land and sea shipping disasters, and other events and activities. Emissions can be from venting storage tanks, blowouts of gas wells, burning petroleum that has been spilled, or burning unwanted gaseous material. Production of crude petroleum presents two major toxicological issues: exposure of animals to the primary raw product and exposure to pollution generated by the production of crude petroleum. Crude petroleum is designated as sweet or sour based on sulfur content, primarily hydrogen sulfide ( $H_2S$ ). Sour petroleum, especially sour gas, is an economic source of sulfur sold as a commodity. Some governments exempt waste generated in oil fields from hazardous waste regulations. In many geographic regions, such as western Canada, the provincial governments own the majority of mineral resources and can require landowners to provide land leases for oil and gas exploitation. Uneconomical products such as gas including  $H_2S$  coming from crude oil and bitumen can be piped to a flare pipe and burned or vented into the atmosphere. Domestic and wild animals are exposed to pollution from oil and gas activity. Issues exist in using agricultural lands for waste disposal and food production. Oil pits also present a risk for wildlife (Trail, 2006).

Oil pits contain waste fluids that may be stored in pits, open tanks, and other sites accessible to wildlife and domestic animals.

Intoxication of animals with crude and refined petroleum does occur. The two major conditions for exposure are spills and leaks of petroleum into aquatic and terrestrial systems. There is an agricultural and petroleum industrial interface from multiple use of lands for agricultural and petroleum production. Land located in remote regions of the world is also used for oil and gas production. Land uses by the petroleum industry include oil and gas well sites, tank battery sites, gathering sites, sweet and sour gas plants, compressor stations, and pipelines. Crude petroleum and chemicals used in the oil fields can be spilled. The dermal, inhalation, and oral routes of exposure can subsequently poison domestic and wild animals. Birds and mammals can have oil soiling their feathers and fur, respectively. Oral ingestion also occurs from dermal exposure and during preening. Loss of insulation protection of feathers and fur can lead to hypothermia. Oil and gas field operations have intentional and unintentional air emissions. The production of sour gas and the removal of sulfur (sour gas processing plants) present toxicology issues. Animals are poisoned when petroleum products are used as medicaments, containers containing petroleum products are used for animal feedstuffs and applications of parasiticides, petroleum containers are left open and unattended, and domestic spills occur during transportation and use. Water can accompany gas and oil to the surface. This is commonly referred to as production water, and it can be high in brine and salt poisoning can occur. Abandoned oil and gas wells can seep petroleum and brine water to the surface.



OIL AND GAS WELL DRILLING  
AND COMPLETING

Drilling

Oil and gas wells are drilled into oil- and gas-producing formations that exist at varying depths below the surface. During drilling operations, drilling fluids are used to cool and lubricate the drill bit, support the walls of the borehole, and move the cuttings to the surface. The weight of the drilling fluid (mud) controls subsurface pressure and aids in preventing blowouts. Different types of drilling fluids and additives are used for drilling the different geologic zones and penetrating the formation. There are different preferences for the composition of drilling fluids, cost considerations, and requirements of the drilling techniques employed. Wide variations in the chemical composition of drilling fluids are found when companies are drilling within the same oil field

(Edwards and Gregory, 1991). Drilling and petroleum companies can be reluctant to disclose substances added to drilling fluids.

Two basic types of drilling fluids used are the water-based drilling fluid and the non-aqueous drilling fluids. Water-based drilling fluids generally consist of salts, bentonite clay, and barium sulfate for density, and other chemicals are added to give desired properties such as lubrication and reduction of foaming (Table 61.1). Brine or freshwater can be used in water-based drilling fluids. Lignite or lignosulfonate, caustic chrome salts, and other compounds (for example, starches and cellulosic polymer) may also be used. In the non-aqueous drilling fluids (invert drilling fluids), freshwater or brine water is emulsified in a hydrocarbon phase. Emulsifiers are used to disperse the water in the hydrocarbons. The water emulsion is generally less than 1 μm in diameter. Diesel fuel is often used as the hydrocarbon in land-based drilling operations. Hydrotreated mineral oil can be used, which has a low content of polyaromatic

TABLE 61.1 Examples of drilling mud additives, substances used to stimulate oil and gas wells, and substances used in the production of oil and gas

Function of substance	Example substance <sup>a</sup>	Function of substance	Example substance <sup>a</sup>
Acids used for well stimulation and other uses	Acetic acid	Breakers, emulsion/gels	Ammonium persulfate
	Acetic anhydride		Benzoic acid
	Benzoic acid		Sodium acetate
	Formic acid		Sodium persulfate
	Hydrochloric acid		
	Hydrofluoric acid	Cement additives	Aluminum (coated)
Add weight to drilling fluid	Sulfuric acid		Calcium chloride
	Attapulgate		Cellulose flakes
	Barium sulfate (Barite)		Cellulose polymer
	Bentonite		Gilsonite
	Galena (lead sulfate)		Gypsum
	Hematite (iron oxide)		Latex
	Siderite (iron carbonate)		Lignosulfonates
Antihydrate (block formation of hydrates)	Jel Chem (e.g., potassium silicate)		Lime
	Alcohols		Long-chain alcohols
	Methanol		Potassium chloride
Biocides	Glycols		Sodium chloride
	Acrolein		Sodium metasilicates
	Anhydrous ammonia	Corrosion inhibitors	Acetylenic alcohols
	Amines		Amine formulations
	Chlorinated phenols		Ammonium bisulfite
	Formaldehyde		2-Butoxyethanol
	Glutaraldehyde		Ironite sponge
	Isopropanol		4,4'-Methylenedianiline
	Methylene bithiocyanate		Paraformaldehyde
	2-(Thiocyanatomethylthio)-1,3-benzothiazole		Sodium chromate
	Thiazolin		Sodium dichromate
	Quaternary ammonium		Sodium metasilicate
			Sodium polyacrylate
			Thiazolin

(Continued)

TABLE 61.1 (Continued)

Function of substance	Example substance <sup>a</sup>	Function of substance	Example substance <sup>a</sup>
Deflocculants	Zinc carbonate	Propping agents	Bauxite
	Zinc oxide		Resin-coated sand
	Zinc lignosulfonate		Sand
			Zirconium compounds
	Acrylic polymer	Salt solutions	Aluminum chloride
	Calcium lignosulfonate		Ammonium chloride
	Chrome-free lignosulfonate		Calcium bromide
	Chromium lignosulfonate		Calcium chloride
	Iron lignosulfonate		Calcium sulfate
	Quebracho		Ferrous sulfate
	Sodium acid pyrophosphate (SAPP)		Potassium chloride
	Sodium hexametaphosphate		Sodium chloride
	Sodium phosphate (oilfos)		Sodium sulfate
	Sodium tetrphosphate		Zinc bromide
	Sodium tripolyphosphate (STP)		Zinc chloride
	Styrene, maleic anhydride copolymer salt		Zinc sulfate
	Sulfomethylated tannin		
Defoaming agents	Aluminum stearate	Scale inhibitors	Ethylenediaminetetraacetic acid (EDTA)
	Fatty acid salt formation		Inorganic phosphates
	Mixed alcohols		Nitrilotriacetic acid (NTA)
	Silicones		Organic phosphates
	Tributylphosphate		Phosphonates
Dispersant – thinner	Causticized metal lignite	Solvents	Polyacrylate
	Ferrochrome lignosulfonate		Polyphosphates
	Sodium tetrphosphate		Acetone
	Lignite		Aliphatic hydrocarbons
	Lignosulfonates		<i>t</i> -Butyl alcohol
Fluoride generators	Ammonium bifluoride		Carbon tetrachloride
	Ammonium fluoride		Chloroform
Lubricants/friction reducers	Acrylamide methacrylate copolymers		Diacetone alcohol
	Graphite		Diesel oil
	Lead grease		Ethylene glycol monobutyl ether
	Lithium grease		Kerosene
	Mineral oil formulations		Isopropanol
	Molybdenum grease		Methyl ethyl ketone (MEK)
	Motor oil		Methyl isobutyl ketone (MIBK)
	Organo-fatty acid salt		Methylene chloride
	Petroleum grease		Methanol
	Reprocessed motor oil		Naphtha
	Sulfonates		1,1,1-Trichloroethane
	Vegetable oil formulations		Toluene
			Turpentine
Multiple uses	Ammonium nitrate		Xylene
Oil-based drilling fluids	Potassium nitrate	Surfactants – emulsifiers	General categories
	Amid polymer formulations		Cationic
	Amine-treated lignite		Non-ionic
	Asphalt		Anionic
	Diesel		Amides
	Gilsonite		Arylalkyl sulfonic acid
	Mineral oil		Fatty alcohols
	Organophilic clay		Glycols
	Organophilic hectorite		Isopropanol
	Petroleum distillate		Modified tall oils (from tree resin byproduct of wood pulp industry)
	Polyethylene powder		Nonylphenol ethoxylates
Oxygen remover pH control	Polymerized organic acids		Oxyalkylated phenolic compounds
	Ammonium bisulfite		Petroleum naphtha
	Calcium hydroxide		Polyamines (diethylenetriamine and triethylenetetramine)
	Potassium hydroxide		
	Soda ash (sodium carbonate)		
	Sodium bicarbonate		
	Sodium hydroxide		

<sup>a</sup>Some items on this list may have historic use and may be in abandoned sumps.

hydrocarbons. Synthetic hydrocarbons and polymers can also be used. Drilling mud can also contain heavy metals, especially aluminum, cadmium, chromium including chromates, copper, iron, lead, mercury, and zinc. Drilling wastes are representative of the formations encountered during the drilling operations. If radioactive materials are encountered in the formations, radioactive materials and gas can be in drilling fluids and drill cuttings.

### Completion and formation fracturing (fracking)

Regulations exist in most governmental jurisdictions to protect groundwater reservoirs. During the drilling of the oil or gas wells, a surface casing is inserted and cemented in place to protect groundwater. Regulations specify the depth that the casing has to extend beyond freshwater aquifers. During the cementing operation, cement is pushed down around the casing, and the hardened cement is the seal between the borehole and the casing. A blowout preventer is attached to the casing. The production tubing is inserted into the borehole and extends from the surface to the oil- or gas-bearing formation. Production of oil and gas is from the tubing. Incomplete or faulty cement jobs and casing failure can result in contamination of the groundwater with crude oil and natural gas.

Oil- and gas-bearing formations are porous, with the oil and gas contained within the pores. To stimulate production, the formation is fractured. In the past, fracturing was done with explosives. Hydraulic fracturing is done by pumping chemicals and solids down the hole and fracturing the formation. A large volume of water (~2 million L) and a variety of substances can be used during fracking operations (Finkel and Law, 2011). Acids can be pumped down the formation to form fractures by dissolving the formation. Propping agents such as resin-treated sand are placed in the fractures to prevent closure.

Oil and gas production can use large quantities of surface and groundwater. The use of water generally increases with depletion of oil in the underground reservoir. Large quantities of water and steam can also be used for *in situ* extraction of bitumen. In both of these operations, water or steam is injected into the formation, sometimes at very high pressure. The water remains in the formation, and oil or liquid bitumen is extracted. The loss of water to underground formations is an issue in the more arid regions of the world, including the western United States and Canada. Arsenic naturally occurring in groundwater can increase as the level of water in the aquifer is decreased when groundwater is pumped out for steam production.

## COAL BED METHANE AND SHALE GAS

The production of coal bed methane can create water issues. Coal bed methane is produced by the *in situ* removal of methane from coal deposits. It is estimated that trillions of cubic meters of coal bed methane reserves exist, making this an attractive source of natural gas. The procedure to produce coal bed methane is to drill a well into the coal deposit and inject liquids under high pressure to hydraulically fracture (fracking) the coal seam (Cobb, 2003; Keith *et al.*, 2003; Young, 2005). After fracking, water is pumped from the coal bed, and the decrease in pressure allows methane to escape into the natural and man-made cleats (cracks in the coal bed). Generally, a larger number of wells are drilled for coal bed methane production than for conventional natural gas production. Coal beds can occur in aquifers. In coal bed methane production, huge quantities of water can be removed from underground aquifers, and aquifer drawdown can affect an area as large as a township. In some regions, the produced water is high in salts (total dissolved solids), and surface water and soil pollution from this water is a significant problem. The saltwater produced in coal bed methane production can be high enough to cause sodium ion intoxication in livestock.

Shale formations are a source of oil and natural gas being exploited by new drilling and fracking technologies. Emerging issues are damage to domestic water supplies and deposits containing radioisotopes.

## SOURCES OF TOXIC SUBSTANCES

Chemicals associated with petroleum intoxication in cattle are the gaseous, liquid, and solid crude petroleum and chemicals associated with the production of natural gas, crude oil, and bitumen, respectively. As it comes from the wellhead, crude natural gas may have volatile liquids and may contain H<sub>2</sub>S and other forms of sulfur, including the polyaromatic thiophenes (Kropp and Fedorak, 1998). Natural gas that contains H<sub>2</sub>S is called sour gas. In addition, a large variety of chemicals are used in the production of crude petroleum. Sour gas can have the H<sub>2</sub>S removed and the sulfur gas disposed of by burning in flare stacks.

### Emissions

Flaring is a method used in the petroleum industry to dispose of unwanted flammable gases. In contrast to an incinerator, a flare produces more products of

incomplete combustion. A flare is essentially burning of gaseous material at the end of a vertical or horizontal pipe. The horizontal flare pipe may end in a pit called a flare pit. Wind and liquids in the flare gas remarkably reduce the efficiency of a flare for burning gaseous waste (Stroscher, 1996). Flaring is done for emergency release of flammable gases, process flaring to dispose of unwanted gases, and production testing. Solution gas is a product derived from crude petroleum when the pressure is reduced to atmospheric pressure. This gas can be flared as an unwanted product. Gas wells are production tested after completion by connection to a flare stack or an incinerator. Production testing is done to remove chemicals from the formation that would corrode pipelines and other equipment and to estimate the economic productivity of a gas well. The gas well is connected to a flare, and the emissions from the well are burned. Stroscher (1996) provided insight into the chemical complexity of flare emissions. Toxic emissions can include a wide variety of combustion products such as chlorinated dibenzodioxins and furans if chlorine and favorable combustion conditions are present (Buckland *et al.*, 2000). Burning sulfur-containing oil field wastes can produce sulfur dioxide (SO<sub>2</sub>), reduced sulfur compounds (including carbon disulfide, carbonyl sulfide, and thiophenes), and polyaromatic hydrocarbons.

## Production water

Production water is water that is brought to the surface during the production of oil and natural gas. Production water can be high in total dissolved solids (TDSs), and ingestion of production water can induce salt (sodium ion) poisoning. Production water can have a unique ratio of ions, and this ratio generally can be distinctly different from TDSs in groundwater. Livestock producers in the more arid regions may use production water that is low in TDSs, and produced water may be lower in TDSs than water from shallow aquifers. Production water can be high in sulfates. Cattle consuming water high in sulfate have increased risk for thiamine-responsive polioencephalomalacia, and the risk increases with decreased dietary copper (Gould, 1998).

## Well rework and pipeline maintenance

A variety of chemicals are used during rework (maintenance of downhole equipment) operations. Spillage of these chemicals occurs, and livestock poisonings have been reported (Monlux *et al.*, 1971). Detergents, surfactants, and other substances can be used during rework operations (Table 61.1). Detergents generally increase

the gastrointestinal absorption of hydrocarbons. Acids, solvents, anticorrosives, and chelating compounds can also be used. Some of these compounds are intrinsically toxic and can enhance the toxicity of other substances. Chelating and descaling agents may increase the bioavailability of chemicals that otherwise would be considered to have a low order of toxicity. Downhole equipment can be laid out at unsecured sites and cattle will lick the petroleum and other substances from the equipment.

Pigging is placing a scraper ("pig") in a pipeline to remove biofilms, wax, and other substances. The pig may contain electronic equipment to inspect the pipeline for corrosion and other defects. A variety of organic and inorganic chemicals, including biocides, solvents, surfactants, and anticorrosive chemicals, can be used in pigging operations. These chemicals are caught in a tank located at an instillation called a "pig trap." Large amounts of these substances can be spilled at a pig trap, and contamination of groundwater used for livestock can occur.

## Sumps and contaminated soil

A sump is generally a pit used for disposal of oil field drilling waste. Many government jurisdictions have outlawed buried sumps. Sump tanks are used and waste is disposed at a proper waste disposal site. The chemical composition of drilling sumps is highly variable and reflects chemicals used in drilling oil and gas wells, heavy metals in formations penetrated by the drilling operation, fracking substances, and other materials disposed of in the sumps (Heitman, 1986; Wascom, 1986). Sump pits vary considerably in size from a small lot to larger than a football field, and they may not be adjacent to oil and gas well sites. Buried sumps can be limited to a series of backhoe trenches. Remote sumps miles from any oil or gas wells have been used for waste disposal. Sump pits can be a ticking time bomb, and environmental issues arise because the current landowner may not be aware that a site exists. Although some governments have progressed in regulating sump pits, old sumps can be a cause of concern (Murphy *et al.*, 1986). A sump can literally be a miniature abandoned hazardous waste site that has defaulted to the legal landowner. Issues arise regarding cleanup of these old sites, and many jurisdictions have laws that declare the legal landowner responsible. Some banks in Canada have given farmland zero value for loans because of old oil and gas well sump pits. In some areas of the world, rough lands with buried oil field sumps have become desirable building sites. Houses and barns have been built on or adjacent to old sumps, and individuals have planted gardens over old sumps. Surface water from rainfall events and melting snow can extrude drilling waste from sumps to the



surface, and these substances can be a toxicology hazard (Mostrom *et al.*, 1993). Domestic and wild animals can be attracted to these areas.

Drill cuttings and spent drilling fluids eventually become wastes and can be disposed of in sump pits. For example, a well 20 cm in diameter and 1000 m deep generates approximately 30 m<sup>3</sup> of drill cuttings. The volume of drilling fluids accumulating in the drilling sump varies from 789 to greater than 160,000 m<sup>3</sup>. The general rule is 0.5 m<sup>3</sup> of drilling waste per meter of depth. Some of the drilling wastes are spread on agricultural lands because of perceived value for the content of nitrogen, potassium, and other plant nutrients. The analytical chemistry of oil field waste can be incomplete and may not reflect the true hazard from spreading these substances on agricultural lands. Issues from this practice include puddling of the drilling fluids and cattle drinking from the puddles. Waste drilling fluids and drill cuttings are disposed of in sumps on the oil lease or at lease sites called remote sumps.

Rain and snow runoff water can extrude contaminants from sumps and contaminated soil. Extrusions from a drilling sump containing invert-drilling fluid have been associated with maladies in cattle (Mostrom *et al.*, 1993). Extrusions from an invert sump contaminated a pasture. Cows grazing this pasture had calves that were stunted. Necropsy findings were abnormal epiphyseal plates in the long bones and myeloid-like bodies in the proximal tubular cells of the kidney (Mostrom *et al.*, 1993). The activities of cytochrome P450 mixed function oxidase enzymes were also elevated. Sheep have been poisoned following extensive rains that caused extrusion of natural gas condensate from contaminated soil (Adler *et al.*, 1992). Valve failure on a condensate storage tank resulted in the soil being contaminated, and subsequent rains extruded condensate from the soil outside the perimeter fence of the lease. Eight ewes were found dead the first day, and the extrusion area was fenced but ewes continued to succumb during a 21-day period. Clinical signs observed were depression, fever, gasoline-like odor to the breath, dyspnea, anorexia, ruminal atony, bloody diarrhea, weight loss, nasal discharge, and recumbency. A gasoline-like odor was detected in ewes necropsied 10 days, but not 18 days, after the last exposure. Cattle grazing in the vicinity of a petroleum complex in India enhanced the presence of As, Ba, Br, Cr, Hg, and Fe in rumen fluid ash, and the elements Na, K, Cl, Cu, Mn, and P were depleted (Garg *et al.*, 1996). The elements were determined by neutron activation.

## Ions

Drilling sumps can also contain high levels of sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>) ions (Darley and Gray, 1988).

Heavy rainfall can extrude potassium salts from old sumps. The extrusions can be confused with whitish discoloration that occurs in naturally occurring alkali areas. When given by oral or intravenous routes, K<sup>+</sup> has an essentially equal order of toxicity (Ward, 1966a). The oral lethal dose of K<sup>+</sup> in a 475-kg cow has been reported to be 238 mg of K<sup>+</sup> (as KCl)/kg body weight (BW) (Ward, 1966b). Because of decreased renal excretion of K<sup>+</sup>, calves are more sensitive to K<sup>+</sup> poisoning than cows, with a lethal dose of 1.73 g of K<sup>+</sup>/kg BW (Blaxter *et al.*, 1960; Ward, 1966a; Neathery *et al.*, 1979). In high doses, K<sup>+</sup> targets contraction mechanisms in cardiac and skeletal muscles. In calves given K<sup>+</sup> by intravenous infusion, changes in the electrocardiograph were observed. With increasing plasma concentrations of K<sup>+</sup>, the QRS interval increases, the P–R interval decreases, P waves decrease, and the Q–T interval becomes very prolonged. With increasing concentrations of K<sup>+</sup> in plasma, the QRS complex becomes disorganized, and atrial flutter, atrioventricular block, and occasional to complete ventricular arrest occur. With increasing concentrations of K<sup>+</sup> in plasma, respiration also increases in rate and amplitude (Bergman and Sellers, 1953, 1954). Altering the K<sup>+</sup>:Na<sup>+</sup> ratio in the rumen has been associated with bloat (Turner, 1981).

Sodium ion poisoning can also occur. Clinical signs are muscle twitching, ataxia, bruxism, aggression, circling, and head pressing. Ingestion of brine water causes dehydration (sunken eyes and loss of plasticity of the skin). Sodium ion in the blood increases, and the electrophysiology of excitable tissues is disrupted. The pathology of Na<sup>+</sup> poisoning in cattle can be variable (Scott, 1924; McCoy and Edwards, 1980; Sullivan, 1985). Ingestion of concentrated Na<sup>+</sup> produces marked congestion of the omasal and abomasal mucosa. Gut contents are fluid, and hemorrhage may cause dark discoloration. Edema of the skeletal muscles and hydropericardium can occur. Edema of the brain can also occur, and an eosinophilic infiltration of the Virchow–Robin spaces may or may not be observed in cattle. Lesions of polioencephalomalacia can also be present. Birds can be poisoned by sodium chloride (Bollinger *et al.*, 2005).

## Lubricant additives

Oil field companies have a substantial investment in surface equipment. This can range from simple reciprocating pumps to complex high-volume pipeline pumping equipment. This equipment can have special lubrication requirements. Manufacturers of specialty lubricants often guard knowledge of the additives in their products as intellectual property. Thus, it can be difficult to obtain this information. It is well known that the *ortho* isomer of the triaryl phosphates is neurotoxic. In North

America, there has been a strong trend away from the neurotoxic tricresyl phosphate additives. However, some of the additives and possibly metabolites produced by microbial degradation can be endocrine disruptors and can target reproductive organs (Somkuti *et al.*, 1987; Latendresse *et al.*, 1993, 1994a). Lesions in the adrenal cortex and ovary have been reported for tricresyl phosphate and butylated triphenyl phosphate. Tricresyl phosphate caused lesions in the testicle, but testicular lesions were not observed with butylated triphenyl phosphate. Both tricresyl phosphate and butylated triphenyl phosphate decreased reproductive performance in male and female rats.

The use of nonpesticide neurotoxic organophosphate esters has been reduced in North America, but these compounds may be buried in disposal sumps (Table 61.2). The neurotoxicity is limited to the *ortho* isomer. Natural gas compressor stations use nonpesticide organophosphate esters to prevent foaming of lubricating oils (Coppock *et al.*, 1995a). Phosphate esters have multiple uses in the petroleum industry, and they have been used in high-performance lubricants. The trivial chemical names describing the industrial grade

generally refer to complex chemical mixtures of these compounds (Dollahite and Pierce, 1969; Beck *et al.*, 1977; Sugden, 1981). For the phosphate esters used in industrial applications, the majority of the individual compounds have incomplete toxicological data (Coppock *et al.*, 1995a). Studies relating the chemical structure of individual tricresol phosphate compounds to neurotoxicity have allowed structural grouping of many of the neurotoxic compounds (Johannsen *et al.*, 1977; Sprague and Castles, 1987; Abou-Donia and Gupta, 1994). The neurotoxicity of organophosphate esters is related to the inhibition of cholinesterases, and the malady produced by inadvertent exposure to triaryl phosphate group (TAP) is known as organophosphate-induced delayed neurotoxicity (Metcalf, 1984). There is wide variation in species sensitivity and the effect of age on sensitivity within species (Abou-Donia and Gupta, 1994). The neurotoxicology of the TAP group of compounds has been described in cattle (Dollahite and Pierce, 1969; Nicholson, 1974; Julian *et al.*, 1976; Beck *et al.*, 1977; Sugden, 1981; Prantner and Sosalla, 1993), other livestock species (Wilson *et al.*, 1954; Dollahite and Pierce, 1969), and chickens (Hixson, 1984). Humans are

TABLE 61.2 Clinical signs of delayed neurotoxicity in cattle

Source	Time sequence <sup>a</sup>	Clinical signs	Reference
Waste hydraulic oil	12 days	Diarrhea	Julian <i>et al.</i> (1976)
	20 days	Posterior weakness, knuckling at fetlocks, ataxia, difficulty in rising, and dog-sitting while attempting to rise	
	20+ days	Diarrhea, coughing, ataxia, difficulty turning, knuckling and buckling of rear limbs, unable to rise but could walk on knees, and decreased sensory and motor response in rear limbs	
	34+ days	Increased severity of clinical signs, incoordination progressing to front legs, standing in a crouched position, muscular wasting, dribbling urine, tail raised, coughing, dyspnea after attempting to rise, and death (14/50)	
Waste oil	NS	Roaring sounds	Prantner and Sosalla (1993)
	2 days	Ataxia of hind limbs and knuckling over at metatarsophalangeal joints	
	42 days	Second heifer, with signs same as for first heifer	
Compressor sump water	7 days	Third heifer, with ataxia, dysuria, polyuria, and dyspnea	Beck <i>et al.</i> (1977)
	42 days	Fourth heifer, with clinical signs same as for third heifer	
	NS	Cows mute when separated from calves	
	7 days	Knuckling of fetlocks, dyspnea, and coughing	
Barrels previously used for hydraulic oils	7 days	Goose-stepping, diarrhea, mute, posterior paralysis, dog-sitting, and recumbency and inability to rise	Nicholson (1974)
	NS	Standing in a crouched position, elevated tails, dribbling urine, and posterior weakness	
	2 days	Posterior weakness, recumbency, and death	
Lubricating – hydraulic oils	NS	Rough hair coats, loss of condition, muscular weakness and incoordination, tympanites, dyspnea, and roaring	Dollahite and Pierce (1969)

NS, not specified.

<sup>a</sup>After exposure.

also susceptible to TAPs (Craig and Barth, 1999). The most common source of TAPs in livestock poisonings results from their use as additives in lubricating and hydraulic oils operating under high pressure and temperature. Cattle will voluntarily ingest water and feed-stuffs adulterated with TAP (Dollahite and Pierce, 1969; Nicholson, 1974; Beck *et al.*, 1977; Prantner and Sosalla, 1993). Oil field-related exposures of cattle to TAP have been primarily from compressor oils and hydraulic fluids (Dollahite and Pierce, 1969; Beck *et al.*, 1977; Coppock *et al.*, 1995a). Compressor lubricating oils in recycled barrels used to store wet molasses were the source of TAP in an incident in which cattle were poisoned (Nicholson, 1974). An undetermined source of waste oil used to lubricate a feed bunk chain was the source of TAP in a poisoning incident in heifers (Prantner and Sosalla, 1993). Waste oil used to treat ringworm was the source of TAP poisoning in a herd of cattle (Julian *et al.*, 1976). Sump pits containing these substances can be uncovered, and cattle, if they have access, may ingest them.

### Antihydrates and antifreezes

Antihydrates and antifreezes are used in the oil fields. Large quantities of methanol are used by the oil and gas industry, and cattle have been poisoned by these sources (Edwards *et al.*, 1979; Rousseaux *et al.*, 1982). Methanol is used to prevent freezing of water in pipes and to prevent hydrate formation in gas wells and pipelines. Intoxication of cattle by methanol has been reported. A herd of 600 cows had access to methanol barrels stored on a lease, and 2 cows were found dead. Manure accumulation behind the cows was taken as evidence that the cows had been recumbent for some time before death (Rousseaux *et al.*, 1982). Rumen contents from the 2 dead cows contained 370 mg of methanol/100 mL of rumen fluid. In another incident, 12/15 cows were found dead or in a moribund condition (Sesevicka *et al.*, 1979). Clinical signs observed were ataxia, decreased rate and depth of respiration, frequent chewing motions, impaired vision, and hypoaesthesia to nociceptive stimuli. These heifers had access to a pit that received wastewater from a gas processing plant. Clinical signs of experimentally induced methanol poisoning in cattle were similar to those reported for field exposures (Fritz and Coppock, 1992). Large quantities of diethylene glycol (DEG) are used in oil and gas operations (Edwards *et al.*, 1979). Research has shown that cattle are a species sensitive to DEG (Fritz and Coppock, 1992; Khan *et al.*, 1992). A dose of 1.5 mL/kg BW is fatal to a cow. Diethylene glycol is toxic to the eye, liver, kidney, and nervous system. Diethylene glycol induced ocular changes that are similar in appearance to those of pinkeye. Diethylene glycol causes a unique lace-like hypertrophy of the

perineuronal amphicytes in the gasserian ganglia in cattle. This lesion has not been reported in laboratory animals. Other glycols are used as antifreezes in oil and gas production.

### Chromate

Cattle are exposed to environmental chromates from oil field sources (Reagor and McDonald, 1980). Gross pathological findings in a calf (8 months old) were icterus, tracheal froth, excessive pleural fluid, and petechiae over the pleura. The intestinal contents were black and mucus covered. Edema and hemorrhage were observed in the mesentery and omentum. Hepatomegaly, yellowing of the liver, and edema of the gallbladder were observed. Histopathological observations included diffuse hepatocellular swelling, vacuolation of hepatocytes, inspissation of bile, and moderate biliary hyperplasia. The kidneys were pale, and hemoglobinuric vacuolation was observed in the cortical tubular epithelium. Proteinaceous exudate was observed in the lung, and epicardial hemorrhages were observed. Chromium concentration in the liver was 14.8 ppm.

## GREASE AND MOTOR OIL

Lithium (Li) intoxication in cattle from Li grease has been reported. Wallace and Blodgett (1996) reported Li poisoning in one cow after the animal consumed Li grease, and Johnson *et al.* (1980) reported on Li poisoning in 19 of 90 animals. In both incidents, the source of Li grease was discarded drums that had been used previously for transporting and storing grease. In the incident reported by Wallace and Blodgett, the cow was euthanized, and in the incident reported by Johnson *et al.*, all 19 animals died. In the animals that consumed Li grease, clinical signs included muscular tremors, ataxia, stiffness, apparent disorientation, ptialism, diarrhea, rapid respiration, and periodic seizure-like activity. The concentrations of Li in grease for the incidents reported were 2050 and 1250 µg of Li/g of grease, respectively. Clinicopathological findings (1 cow) included leukopenia, lymphopenia, and hyperfibrinogenemia (Wallace and Blodgett, 1996). The parameters of protein, cytology, and glucose in cerebral spinal fluid were normal. An oral dosing study with Li has been done in cattle (Johnson *et al.*, 1980). Cattle were administered single oral doses per gavage of 250, 500, or 700 mg of LiCl/kg BW. The LiCl was in water (qs to dissolve the Li) plus an equal volume of water to rinse the stomach tube. Clinical signs were reported (Table 61.3). The most consistent finding

TABLE 61.3 Summary of clinical signs of Li intoxication in cattle

Oral dose (mg/kg)	Time	Clinical signs
250 (4) <sup>a</sup>	2–6 h	Ptyalism
	4–6 h	Depression, anorexia, and diarrhea
	72 h	Diarrhea
	9 days	Recovered
500 (5)	3–4 h	Ptyalism (lasted for 4–6 h)
	After 4–6 h	Depression, anorexia, hypodipsia, anuria, and diarrhea
	7 days	4 animals died
	11 days	1 animal died
700 (4)	2 h	Ptyalism (continued for 4–6 h)
	After 2 h	Depression, anorexia, hypodipsia, anuria, ataxia, and severe diarrhea
	8 h to 7 days	Deaths occurred

<sup>a</sup>Numbers in parentheses indicate the number of cows in the trial.  
Data from Johnson *et al.* (1980).

at necropsy was gastroenteritis of varying severity. Histopathological findings were congestion of the gastrointestinal tract. Lesions in the liver included cloudy swelling, edema, and cirrhosis in the portal triad areas. Renal lesions included cloudy swelling of the proximal tubular cells and a mild interstitial nephritis. Lithium can also be present in process water.

Grease and motor oil generally contain additives and other metallic substances that provide water resistance, increase lubrication under high pressure, and provide other desirable properties. Grease containing lead has excellent water-repellent properties. Lead grease is also a hazard to livestock. Molybdenum is added to grease to improve lubrication under extreme pressure. Motor oil contains additives to prevent foaming, keep carbon deposits in suspension, and reduce friction and wear of parts at high temperatures. Motor oil contains additives to improve lubrication under low and high temperatures, pour-point improvers, and antioxidants. Synthetic oils can contain molybdenum and surfactants. Antiwear additives such as zinc dialkyldithiophosphate compounds and derivatives are added to lubricants to reduce friction. Micronized metals can be added to lubricating oils. Many additives are mixtures of substances, and most companies closely guard their intellectual property.

## TOXICOLOGY OF CRUDE AND REFINED PETROLEUM

Ruminants are attracted to and ingest petroleum. They like it and ingest it. The most important short-term risk

TABLE 61.4 Chemical characteristics of crude oils used in the Rowe and Bystrom studies

Fraction	Pembina crude <sup>a,b</sup>	Texas sweet (%) <sup>c</sup>	Texas sour (%) <sup>c</sup>
Light gasoline	NA	14.2	9.6
Total gasoline or total naphtha	22.1%	43.0	31.6
Kerosene	15.3%	22.3	4.7
Lubricating	NA	8.8	16.8
Sulfur	0.24%	<0.1	1.5
Nitrogen	0.1%	0.083	0.074
Nickel	2.53 ppm	NA	NA
Vanadium	1.17 ppm	NA	NA

NA, not available.

<sup>a</sup>Pembina Cardium crude from Drayton Valley, Alberta, Canada.

<sup>b</sup>Mass percentages.

<sup>c</sup>Units in terms of percentage mass or percentage volume were not given.

for ingestion of crude oil, diesel oil, and kerosene is chemical pneumonia. The risk of developing pneumonia is greatly increased by the occurrence of emesis or nervous system dysfunction. Nervous system dysfunction is caused by volatile hydrocarbons. Weathering of crude petroleum reduces the occurrence of signs of nervous system dysfunction. Milk is tainted after the ingestion of crude and refined petroleum.

## Experimental studies

The toxicology of petroleum has been reported for experimental studies. Rowe and colleagues (Rowe, 1972; Rowe *et al.*, 1973) found that sweet crude oil was more toxic in cattle than was sour crude oil (oil that contains sulfur) (Table 61.4). Administration of 8 mL of sweet crude oil/kg BW/day caused death within 7–14 days in 4 of 5 calves. For the same dose of sour crude oil, 5 of 5 calves died between days 16 and 24; for kerosene, 5 of 5 calves died between days 9 and 23. Administration of 37 mL of sweet crude oil/kg BW was fatal within a few minutes. The majority of the surviving animals had pneumonia.

Bystrom (1989) studied the acute effects of crude oil in cattle. The dosage levels (administered per gavage) were 20, 40, 60, or 80 mL of sweet crude oil/kg BW, or 80 mL of potable water/kg BW. The analytical characterization of the oil is given in Table 61.4. Following exposure, daily mean rectal body temperatures were normal. Ruminal motility was decreased after animals were dosed with oil and slowly increased to pretreatment values by day 8. Vomiting was not observed in the animals dosed with water. Vomiting occurred in 10 of 12 of the animals



dosed with oil, and in some animals, vomiting was characterized by a projectile expulsion of oil. Other animals had oil dripping from their nostrils. Emesis recurred after forage or water was ingested. Ruminant tympanites was observed in the majority of the animals but did not require medical intervention. Neurological abnormalities were not observed in control animals. Central nervous signs expressed by nystagmus, muscular tremors, and petit mal-like seizures were observed in treated animals. Depression was the most common clinical sign observed. These effects were attributed to the anesthetic-like effects of the volatile hydrocarbons. The neurological effects contributed to aspiration pneumonia. Oil in feces was determined by gas chromatographic methods. Oil was found in feces at 23h after dosing for the 20- and 40-mL/kg groups, at 7–31h for the 60-mL/kg group, and at 5–19h for the 80-mL/kg group. Oil was not detected in feces from the control animals. Using head-space (space between the liquid and stopper) analysis, it was demonstrated that volatile constituents from the oil were present in blood. *N*-heptane was used as a representative hydrocarbon. Semiquantitative values for total light naphtha were estimated to be as high as 10mg/L of blood. Aspiration of oil into the lungs increased the levels of *N*-heptane in the blood.

Considerable variation in the dose–response of cattle to mineral petroleum has been reported. These effects can be due to the presence of surfactants or emulsifiers and other additives in refined petroleum. A 200-mL single oral dose of odorless kerosene administered to a mature dairy cow caused a 90.3% reduction in dry matter intake, and the same dose of Stanvac Odorless Solvent caused a 42.5% reduction in dry matter intake (Reid, 1957). These effects were considered to be in remission by day 3 of the study. Emulsification of kerosene with a non-ionic surfactant decreased the interval between exposure and the onset of effects. Similar findings were observed when kerosene was placed directly into the omasum. Fistulation of the rumen did not alter the response to kerosene or solvent.

Exposure to petroleum can reduce body stores of fat-soluble vitamins. McDowall *et al.* (1957) administered 75mL of heavy liquid paraffin/cow/day by drench for 26 days. A 40% reduction in blood carotene occurred over 16 days, and a 20% reduction in blood vitamin A ester and a 40% reduction in tocopherol (vitamin E) were also observed. After cessation of treatment, 21 days were required for recovery to pre-exposure levels.

## Ingestion of petroleum

In the scientific literature, there are numerous reports of cattle and other animals voluntarily ingesting petroleum and other oil field substances (Coppock *et al.*,

1986a, 1995b). Cattle are attracted to and will ingest several gallons of petroleum (Monlux *et al.*, 1971; Oehme, 1977). Deaths have occurred after cattle drank tractor paraffin and vaporizing oil (Eaton, 1943). Heifers drank gasoline (Albert and Ramey, 1964), and Messerli (1969) reported that cattle greedily ingested diesel oil flowing from a storage tank. Cattle have drunk from petroleum puddles near a tank battery, from slush pits, and from puddles of volatile petroleum and petroleum distillate (Edwards *et al.*, 1979). Cattle have consumed crude oil spilled by a pipeline break and have drunk from puddles of road oil after it was applied for dust suppression (Coale, 1947; Bumstead, 1949). Cattle will drink used motor oil (Gardner, 1977) and will also ingest petroleum-contaminated forage (Stober, 1962; Beck *et al.*, 1977). An entire herd of heifers was irreversibly poisoned by ingesting water from a stream that had been previously contaminated with aviation turbine fuel (Barber *et al.*, 1987). Monlux *et al.* (1971) reported that the majority of animals avoid oil but that, in some instances, the entire herd, especially feeder calves, is attracted to oil. Cattle have ingested sump oil with adverse effects on health (Ballantyne, 1950, 1955). Other ruminant species also ingest petroleum. Sheep have ingested Bunker “C” fuel oil (a residual fuel oil) following the sinking of a tanker ship (MacIntyre, 1970). Ingestion of surface water contaminated with extrusions by rainwater of natural gas condensate has caused fatalities in sheep (Adler *et al.*, 1992). Goats voluntarily ingested kerosene (Pathan, 1961). In Iran, goats ingested diesel fuel leaking from an overturned truck tanker (Toofanian *et al.*, 1979).

## Experimental studies on ingestion of petroleum

Experimental studies on the ingestion of crude oil by cattle have been reported (Rowe, 1972; Rowe *et al.*, 1973; Coppock *et al.*, 1992). Calves were observed to drink sweet and sour crude petroleum. After water had been withheld for 48h, calves drank 4L of either a sweet or a sour crude oil, but they would not repeat the ingestion of crude oil after water had been withheld for a total of 96h. Two of the calves that drank oil had been administered per gavage kerosene in a previous study. Calves that were not water deprived did not drink oil. These findings suggest that cattle may acquire some aversion to drinking crude petroleum, and water deprivation can be a factor in the ingestion of kerosene. An experimental study demonstrated that cows on a balanced diet and provided water *ad libitum* are attracted to crude mineral petroleum and will ingest it (Coppock *et al.*, 1992). In a study on voluntary ingestion of crude oil, a stall was designed to minimize competition at the oil source. All of the animals explored the oil, and 5 of 10 animals ingested oil. For 3 of 4 animals that drank oil,

TABLE 61.5 Clinical signs of acute petroleum poisoning in cattle

Acute signs (<24h)	Subacute signs (>24h)	Chronic signs (>30 days)
Ruminal tympanites <sup>a</sup>	Depression	Lethargy
Emesis	Pneumonia	Varying degrees of anorexia
Postural weakness	Anorexia	Loss of condition
Ataxia and incoordination	Constipation	Impaired reproductive performance
Seizures	Coughing	Chronic cough
Hyperthermia	Ileus	Abortion
Dyspnea	Recumbency	Laminitis
Bloody diarrhea	Ruminal atony	Loss of body weight
Strong petroleum-like odor on breath; feces have odor of petroleum	Abortion	Pain – hardware-like signs
Ruminal atony	Loss of weight	
Depression	Sweet petroleum-like odor to breath and feces; feces have petroleum odor	
	Lethargy	

<sup>a</sup>Ruminal tympanites is not a consistent finding.

ingestion occurred most frequently in the first few days during which the oil was available. The most frequent method of ingesting oil was by licking; the cyclic pattern suggested that cattle go on oil-licking “binges” in a substance abuse pattern. The neurochemical reasons for the attraction of cattle to oil are not known. This study showed that cattle provided water *ad libitum* and receiving a balanced diet do ingest crude oil.

### Clinical observations and findings

Varied clinical signs have been observed in field incidents of petroleum poisoning (Table 61.5). A herd of 58 yearling steers had access to crude petroleum distillate (condensate) on an oil-lease property (Edwards and Zinn, 1979). Following oral exposure, 17 animals became sick and 9 died. The sick cattle had petroleum distillate dripping from the nostrils, and it was present in their feces; they also had petroleum on their tails and rear quarters. Surviving animals had varied signs, including anorexia and weight loss. Some of the more severely affected animals died. Edwards and Zinn also described clinical signs in 18 of 135 animals that had access to a sump pit. The surviving animals lost weight and were unthrifty (“poor-doers”). These authors also reported that of 200 steers with access to petroleum in puddles, 12 were found dead and 13 died 24 h later. Oehme (1977) attributed abortions to ingestion of petroleum products. Loss of body condition can also result from petroleum ingestion-linked chronic pneumonia and pleural adhesions. Clinical evidence of pain can be associated with pleural adhesions (signs similar to those of hardware disease). Cattle attracted to an area saturated with condensate can have laminitis-like clinical signs.

A herd in Alberta, Canada, had access to sump oil (Ballantyne, 1950). A 545-kg cow, representative of a malady observed in 20 other cows from a large herd, was necropsied. After ingestion of oil, this cow had a reduction in body weight. Necropsy findings were as follows: there was visible oil in the rumen, the mucous membranes of the abomasum were stained black, and areas of inflammation were observed in the gut. There was 213 mL of oil/L of rumen contents. In another report, cattle drank from a pool of oil (Bumstead, 1949). Two animals died, and all of the animals that had wandered into the pool of oil showed clinical signs of intoxication. One heifer had severe enteritis, was dehydrated, and had cachexia. All of the animals treated by the veterinarian were considered to have made a complete recovery, which was attributed to treatment that rapidly removed oil from the digestive tract and prophylactic treatment with an antimicrobial chemotherapeutic. In another report, 5 dairy cows drank crude oil that had escaped from a broken pipeline. All 5 of the animals were sold 90 days later because of lost productivity (Coale, 1947).

Gibson and Linzell (1948) reported that dairy cows drank petroleum after 5 gallons (23 L) was mistakenly dumped in a water trough. Clinical signs in 6 of 8 cows were central nervous system (CNS) depression including coma, coughing, salivating, head shaking, hypothermia, and petroleum odor on respired air and milk. The diesel-like odor on the breath and in the milk persisted for 5 days. Three animals died at 12, 24, and 48 h, respectively, after exposure. During the interval between exposure and death, all of the animals ate and ruminated but were dull in temperament and were constipated. Forty-eight hours after ingestion, 2 cows had abnormal respiratory sounds, which in 1 cow persisted

for 14 days. The pathological findings were chemical pneumonia and evidence of inflammation of the mammary gland. Another author reported clinical signs of vomiting and death after a lactating Shorthorn animal ingested approximately 1 gallon (4.5L) of tractor paraffin (Eaton, 1943). Twenty-four hours after ingesting tractor paraffin, a 3-year-old bullock had signs of marked excitement to wildness, blindness, and incoordination. Clinical signs of anorexia and constipation were reported after cattle ingested tractor vaporizing oil (low grade of kerosene) (Parker and Williamson, 1951). Pathological findings were fatty changes in the liver, degeneration of the kidney, and multiple small hemorrhages in the lungs. Twenty-four dairy cattle ingested diesel oil from a storage tank valve that was opened, possibly by the cattle (Messerli, 1969). Clinical signs of intoxication were observed in 9 of 24 cows 30h later, and 4 of 24 cows were considered to be seriously affected. Milk from all the cows had a diesel odor. From this herd, a Brown Swiss cow in critical condition was examined in detail. Clinical signs observed in the cow were elevated body temperature, bradycardia, decreased appetite, decreased peristaltic ruminal movements, diarrhea, muscular weakness, and decreased milk production. Eight days after ingesting diesel, swelling of the rear fetlocks was observed. Eighteen days after ingesting oil, the herd milk production was diminished by 75%; 2 months later, unsteady movements were observed, and after an unspecified interval, the cow regained her health. Three of the other seriously affected animals required 1 month for recovery and had retarded growth for a substantially longer interval. The feces had a diesel odor for 5 days.

Peterson (1963) reported that after a herd of cattle had access to heating oil-contaminated water for 12h, two cows showed remarkable clinical signs. One cow was pyrexia (41.1°C) and had severe dyspnea. The body temperature of the second cow was 38.8°C, and there was clinical evidence of abdominal pain. The first cow was treated with intravenous antibiotics and laxatives; she passed fuel oil in her feces and made a complete recovery. The second cow received similar treatment, did not eat for 2 weeks, developed septic metritis followed by pneumonia, and subsequently died. Poor health and an abnormal liver in a heifer were attributed to her drinking gasoline 15 months previously (Albert and Ramey, 1964). Immunosuppression could have occurred, and no mention was made of plumbism.

Barber and colleagues (1987) reported an environmental incident with aviation turbine fuel. Fifty-one heifers 12 to 18 months old were exposed to water in a stream that had been contaminated with aviation turbine fuel. The duration of exposure was not determined and, when discovered, the contaminated waterway was immediately fenced and the heifers

were provided an alternative source of drinking water. Observations at the initial appointment were 2 heifers dead and 5 animals with signs of ataxia. During a 3-week interval, 8 heifers died or were euthanized *in extremis*. Animals that survived were dull (CNS depression), did not show interest in strangers walking about the field, and had remarkable weight loss, and increased respiratory rate was observed in some animals. Six weeks after the initial examination, 41 of the surviving animals appeared unhealthy. At 121 days after the initial examination, the heifers were compared to siblings and were found to have lost 50–100kg. Because of apparent chronic, poor health, 41 of the heifers were slaughtered approximately 124 days after the initial exposure. One heifer died during transport to slaughter. After slaughter, the animals were submitted for pathological examination. All of the heifers were poorly fleshed for their skeletal size. Some of the animals had liver flukes, but lungworms were not observed. Essentially all of the lungs were considered to be grossly abnormal, as exemplified by enlargement and grayish-blue areas of varying size, and had firm consistency. However, histopathological abnormalities were not observed in the lungs and kidneys.

### Dermal toxicity of petroleum

Pesticides applied to cattle using “cattle oilers” are often diluted with diesel oil and kerosene. Petroleum distillates are sometimes used as vehicles for fly sprays. Diesel fuel was considered to be the cause of a severe skin reaction to fly spray (Edwards and Niles, 1981). The abdomens and legs of the cows were sprayed each time the cows entered the barn, and when the cows exited the barn, the same mixture was rubbed on their backs. The fly spray was a mixture of 3.8L of proprietary fly spray mixed with 53L of diesel fuel. Erythema of the skin, swelling of the carpus, and varying severity of edema were observed on the front legs between the elbow and fetlocks. Skin on the back, rear legs, and udder was not affected. The authors postulated that washing before milking protected the udder and rear legs. The fly spray–diesel mixture produced similar lesions on the backs of guinea pigs. Treatment consisted of washing the front legs, application of scarlet oil, and discontinuing the use of the fly spray mixture. Using a different source of diesel fuel for diluting the fly spray was considered significant in causing the dermatitis. The causative agent in the diesel fuel was not identified. The mixture of pesticide and kerosene and diesel oil can increase the rate of absorption of pesticide. The diesel oil–pesticide mixture can be spilled from overturned oilers. Cattle were attracted to soil that had been saturated with crude condensate. Puddles of condensate were observed in the

cattle tracks. Clinical signs of laminitis were observed in many of the animals in this herd. It is assumed that the petroleum was absorbed through the hoof wall and caused inflammation.

### Biochemical toxicology

Studies on the biochemical toxicology of crude and refined petroleum have been performed (Khan *et al.*, 1989). Initial studies showed that Pembina Cardium crude oil (PCCO) increased hepatic microsomal protein cytochrome P450 content and the activities of NADPH-cytochrome *c* reductase, aryl hydrocarbon hydroxylase (AHH), 7-ethoxycoumarin-*O*-deethylase (ECOD), and cytosolic glutathione transferase. The clinicopathologic and biochemical effects of PCCO or commercial diesel fuel (CDF-2) were studied in rats (Khan *et al.*, 2001). On days 1, 3, 5, and 8, specified dosages of the control vehicle, methylcellulose (MC) (1.25 mL/kg), PCCO (0.25–1.25 mL/kg BW), or CDF-2 (1.25 mL/kg BW) were given per gavage. No overt signs of intoxication were observed. Liver somatic index increased in rats exposed to 1.25 mL/kg doses of PCCO and CDF-2. Hematology and serum chemistry parameters were not significantly increased. The PCCO- and CDF-2-exposed groups had a significant increase in the hepatic activity of ethoxyresorufin-*O*-deethylase (EROD). In PCCO-exposed rats, the induction of EROD increased with dose. Dose-related increases occurred in hepatic activities of AHH, ECOD, glutathione transferase, and NADPH-catalyzed microsomal lipid peroxidation. The renal activity of ECOD was significantly increased. Studies in rats have shown that these enzymes downregulate in a temporal manner after per gavage exposure to low levels of PCCO are discontinued (Khan *et al.*, 2002). Cattle were administered single oral doses of PCCO at 16.7, 33.4, and 67.4 g/kg BW or 80 g water/kg BW (control) (Khan *et al.*, 1996). Cattle from each treatment group were sacrificed on post-treatment day 7 or 30, and biochemical parameters were assayed in liver, lungs, and kidney cortex. On day 7, the PCCO-treated groups showed marked alterations from the control group in hepatic cytochrome P450 and in the activities of AHH and ECOD. There was a 100% increase in hepatic cytochrome P450. AHH activity was increased in all tissues. The enzyme activity was increased greater than 5000% in kidney cortex, greater than 500% in liver, and greater than 250% in lungs. The activity of ECOD was increased greater than 1300% in kidney cortex, slightly increased (20–30%) in liver, and decreased (>80%) in the lung parenchyma. The activities of respiratory chain enzymes were not changed in any tissues. By day 30, the alterations in cytochrome P450, AHH, and ECOD observed on day 7 were markedly reversed.

## VETERINARY MEDICAL AND PESTICIDE USES OF PETROLEUM

### White oils

Mineral oils are purified and used for medical purposes and added to human foodstuffs and animal feedstuffs (IARC, 1984). The mineral oils are highly refined naphthenic or paraffinic distillates. The unsaturated compounds, including aromatics that add color and taste, and sulfur compounds have been removed. Treatment methods including solvent extraction and steeping with strong sulfuric acid can be used as well as adsorption with clay minerals. Alternatively, hydrogenation can be used. To meet U.S. Pharmacopeia (USP) standards, mineral oils have a very low sulfur and aromatic and polyaromatic hydrocarbon content. Mineral oils are used in the food industry, used as lubricants in manufacturing of human foodstuffs, directly added to foodstuffs, and used as anti-bloat oils and a pharmaceutical. Purified mineral oils are mixed with surfactants (pasture spray oil) and sprayed on alfalfa (lucerne) forage before grazing as a prophylactic for bloat in cattle. Food-grade mineral oils are used as horticultural oils to control pests (Beattie *et al.*, 2002).

### Kerosene and diesel oils

There is historical record of various mineral petroleum fractions being used as veterinary medicaments. Lamp paraffin (kerosene) and other kerosene-like products were used as a veterinary medicament for treating animals with intestinal helminths, frothy bloat, and diarrhea, and crude oil was used as an aperient. The unpredictable adverse reactions of orally administered kerosene are anorexia, ptyalism, indigestion, irritation of the digestive tract, respiratory difficulties, and death. These effects were attributed to its variability in chemical composition (Reid, 1957; Stober, 1962). Clinical signs have been described for a cow that was drenched with lamp oil. In the first incident (Shenton, 1937), the owner administered 1 pint (0.57 L) of paraffin oil to control bloat. Twenty-four hours later, the cow was examined and a rumen cannula inserted. Forty-eight hours later, there was an absence of rumen motility, and the cow would vomit after drinking water. On day 3, a stomach tube was passed, and gas and water were removed from the rumen. On day 4, the cow drank gruel and water, and it died on day 5. Necropsy findings were severe inflammation and necrosis of the esophagus, inflammation of the abomasum, and the smell of paraffin in the ingesta. Munch (1956) advised that because of unpredictable adverse effects, crude oil should not be



used as a laxative for cattle. Exposure to mineral oil can deplete fat-soluble vitamins. McDowall (1957) administered per gavage 75 mL of heavy paraffin/cow/day by drench for 26 days. A 40% reduction in blood carotene occurred over 16 days, and a 20% reduction in blood vitamin A ester and a 40% reduction in vitamin E were also observed. After cessation of treatment, 21 days were required for recovery to pre-exposure levels.

## SOUR GAS

Sour gas is natural gas that contains  $\text{H}_2\text{S}$ , other sulfur compounds, methane, and other petroleum hydrocarbons. In addition to the toxicity of  $\text{H}_2\text{S}$  and other sulfur compounds, sour gas is extremely irritating to the eyes and respiratory tract. Sour gas wells are drilled and produced for natural gas (methane), sulfur, and liquids (a variety of commercially valuable hydrocarbons). The crude sour gas is collected in a pipeline system ending at the sour gas plant. At this facility, production water, liquids, and the sulfur are removed. Removal of sulfur compounds is referred to by the petroleum industry as the sweetening process. Release of sour gas and other sour products occurs as fugitive emissions, pipeline breaks, wellhead incidents, and from unlit flare stacks. Sour gas is a complex mixture of chemicals, and the chemical composition varies from well to well. It is likely that indexing the toxicity of sour gas on  $\text{H}_2\text{S}$  levels underestimates the toxicology. Stress from irritation of mucous membranes and other factors can increase the risk of infectious pneumonia in cattle and horses.

### Sour gas well blowouts

Sour gas well blowouts constitute a unique hazard to livestock. In the blowout incidents that have been reported in the literature, cattle were confined by fences, corrals, etc. and generally could not escape the airborne emissions. Ranchers and their families may have been under emergency evacuation order and forced to leave their cattle and other livestock unattended. The evacuation alone can cause major disruptions in animal husbandry. Sour gas is heavier than air and settles into low areas, especially during cool temperatures. Low areas tend to be sheltered from the wind, and less mixing of the air occurs. The toxic effects of sour gas observed in these incidents are summarized in Table 61.6. Details of the incidents are provided.

During rework operations of a sour gas well in western Canada, a blowout occurred (EPS, 1973). The rate of flow and  $\text{H}_2\text{S}$  concentrations were estimated at  $11.6 \text{ ft}^3/\text{s}$

and 4–8%, respectively. Ideal conditions for the dispersal of gas were reported to have existed during the blowout. Cattle in one herd were reported to have a watery discharge from eyes and nostrils. The Veterinary Diagnostic Laboratory examined cattle from six farms. The diagnosis was pneumonia.

Cattle were studied in western Canada after a sour gas well blowout (Lodgepole blowout) that occurred in 1982 (October 17 to December 23). The duration of the blowout was essentially 67 days, and the estimated flow rate was greater than  $4.2 \times 10^6 \text{ m}^3/\text{day}$ , giving an estimated release of 900–1400 metric tons of elemental sulfur equivalent/day. The well was ignited at 14:07h on November 1, 1982, and burned until November 17. The well was ignited again at 12:53h on November 25, 1982, and burned until it was extinguished during the capping process. The well was not on fire for 27 days, and it was ignited for approximately 40 days. Two human fatalities occurred during attempts to control the well. There were differences of opinion concerning the harmful effects of emissions from the Lodgepole well. Round (1992) reported clinical signs of ocular and respiratory irritation that was locally described as “red-eye syndrome.” Improvement of cattle with the red-eye syndrome was observed immediately after the well was capped. The well emissions were considered to be suspect in causing abortions, and infectious causes of abortions were not identified. The number of aborted fetuses and diagnostic trends were reviewed for the interval before, during, and after the Lodgepole blowout (Klavano and Christian, 1992). Concerning submissions sent to the Animal Health Laboratory, the Lodgepole blowout did not result in a change in the number of fetuses submitted to the laboratory or the diagnostic trends established for aborted fetuses. A study was done on the attitudes and opinions of livestock producers regarding the long-term effects in cattle of the emissions from the Lodgepole blowout (Harris, 1992). The producer-based observations were on 1700 beef and 40 dairy cows. Problems associated with trace mineral deficiencies, including change in hair color, were more prevalent among exposed cattle; complaints of blowout-associated maladies in cattle increased with increasing proximity to the well. Complaints of blowout-associated maladies also were more common for cattle located in low-lying areas. Concerns regarding low birth weights were more common for producers who had not recorded birth weights. Other problems included difficult calving due to *inertia uteri*, calves born with deformed feet, and calves that were “poor-doers.” Farmers complained of reduced growth in replacement heifers. The study included one dairy producer. Lactating cows exposed to the blowout gas had a 20% reduction in milk production. There was no report of decreased milk production in cows that freshened after the blowout. The calving interval

TABLE 61.6 Summary of observed effects of blowout emissions in cattle

Blowout	Observations	References
Canada	Watery discharge from eyes and nostrils; pneumonia was observed.	EPS (1973)
Canada, Lodgepole blowout	Ocular and respiratory irritation, abortions, and other reproductive problems.	Round (1992)
	Number of aborted fetuses and diagnostic trends did not change for the Provincial Laboratory (Edmonton) during and after the blowout.	Klavano and Christian (1992)
	Maladies in cattle increased with decreasing distance from the well and were increased in geographically low areas; problems associated with trace mineral deficiency were observed, as were changes in hair color; <i>inertia uteri</i> , calves were born with deformed feet; calves had failure to thrive, decreased growth of replacement heifers, possible decreased birth weights, and 20% reduction in milk production by dairy cows; calving interval increased; with increased interval from exposure, the herd returned to normal.	Harris (1992)
	Significant reduction in weaning weights of calves exposed as cow-calf pairs.	Whitelock (1992)
	Possible association with exposure to well emissions were as follows: Physical examinations were within the expected normal variation; pathological findings were of varied diagnosis and were not considered to be unique; three calves from these cows raised at the laboratory grew at average to above average rates; parameters used may not have been sensitive enough to detect irreversible toxicological effects; and the owner may have been biased in his evaluation of long-term effects.	Church (1992)
Mississippi (USA)	Irritation of the eyes and respiratory tract; respiratory distress; clinically affected 9 months later.	Edwards (1992)
Canada, Drummond	Investigation team concluded that reasonable evidence may have existed for transient respiratory disease, extended feeding time to finish exposed feedlot cattle. Possible for transient exercise intolerance in horses to be linked to exposure to sour gas. Probable links to eye and respiratory irritation in cattle and horses.	Anonymous (1986)
Canada, multiphase pipeline leak	Ocular and nasal irritation; evidence of immune and nervous system dysfunction; aggressive behavior in cows; <i>in estrus</i> behavior of pregnant cows; above average mortalities in cows and calves; calves lacked sucking instinct; cows failed to nurture newborn calves; lymphoid and thyroid hypoplasia and putative endocrine disruption.	Mostrom <i>et al.</i> (1995), Mostrom and Campbell (1996)

increased to 13.9 months during 1983 and then returned to 11.8 months the subsequent year. The owner considered his herd to have been affected by the blowout emissions and then returned to normal.

Whitelock (1992) reported his observations in cattle attributed to the emissions from the Lodgepole blowout. He observed a statistically significant reduction in weaning weights after exposure of cow-calf pairs and weaned calves to emissions from the Lodgepole well. Church (1992) interviewed farmers and evaluated cattle for toxic effects. In a 118-cow herd of purebred Angus cattle exposed to the well emissions, eight parameters may have been related to exposure to blowout emissions: (1) average decrease of 15 pounds (7kg) in birth weight, (2) a 100-pound (45-kg) decrease in weaning weights, (3) a 4.6% increase in birth defects or stillborn calves, (4) a 9.5% increase in abortions, (5) loss of hair color (fading and graying), (6) decreased growth and milking ability of heifers born in 1983, (7) breeding problems such as abnormal cycling, and (8) a 23% increase in culling rate. Three of 10 control herds had two of the problems listed for the exposed herd. One of the farmers requested that his cows be evaluated by laboratory procedures (Church, 1992). Six cows, considered by the owner to be the worst

affected, were examined in 1986. The laboratory findings were as follows: (1) three calves from these cows raised at the laboratory grew at average to above average rates, (2) findings of physical examinations were within the expected normal variation, (3) pathological findings were of varied diagnosis and were not considered to be unique, (4) parameters used may not have been sensitive enough to detect irreversible toxicological effects, and (5) the owner may have been biased in his evaluation of long-term effects.

Edwards (1992) described the impact of emissions in cattle from a sour gas well blowout that occurred in 1985 in the southern United States. The well blew for 72 days at a pressure of 1340 kg/cm<sup>2</sup> (19,000 pounds/in.<sup>2</sup>); the well produced 1.1 million m<sup>3</sup> (40 million ft<sup>3</sup>) of emissions/day. The emissions were ignited because the gas was composed of 35% H<sub>2</sub>S. A herd of red and gray Brahman was examined 5 days after the blowout started. Clinical findings were irritation of the eyes and respiratory tract. Of the 55 animals with signs of respiratory distress 25–30 days after the blowout started, 15 remained clinically affected approximately 9 months later.

A sour gas well blowout occurred in western Canada in 1984, and unrestricted emission of sour gas occurred

TABLE 61.7 Chemical composition of the Drummond 6-30 well

Compound	Concentration (ppm)
Hydrogen sulfide	11,400
Methyl mercaptan (methanethiol)	57.0
Carbonyl sulfide	22.0
Ethyl mercaptan (ethanethiol)	14.7
Propyl mercaptan	14.3
Carbon disulfide	4.8
Butyl mercaptan	3.3
Dimethyl disulfide	<0.5

for 88 h (Anonymous, 1986). Flow rates for the well were estimated at 239,000 m<sup>3</sup>/day. The approximate concentration of sulfur-containing compounds is given in Table 61.7. A total of 195 measurements of H<sub>2</sub>S were made with mobile monitors in the blowout area; the concentrations ranged from 0.014 mg of H<sub>2</sub>S/m<sup>3</sup> of air to 4.90 mg of H<sub>2</sub>S/m<sup>3</sup>. Monitoring of H<sub>2</sub>S did not occur until 5 h after the blowout. The blowout occurred on September 25, 1984. A team of veterinarians conducted an investigation into possible effects of the well emissions on livestock health. Sixteen ranches were visited, and 5 ranches received detailed investigations. General complaints consisted of irritation of ocular and respiratory membranes, respiratory disease (pneumonia), reduced exercise tolerance, and reproductive failure. A ranch located approximately 2 km from the well had 151 head on a fattening ration. These animals were exposed to unknown levels of sour gas in the first few hours after the blowout. Subsequently, mobile monitors recorded 1.0–50 ppm H<sub>2</sub>S in the vicinity of the cattle. Mobile monitoring with handheld monitors was done primarily for access to the area under emergency management controls. Subsequent to the exposure to sour gas, the majority of the 151 head of fattening steers showed irritation of ocular and nasal membranes and had abnormal lung sounds and exertional dyspnea. The signs suggested an impending outbreak of infectious respiratory disease. However, these signs abated without treatment over a period of several weeks. One animal was necropsied and found to have necrobacillosis. Another animal had CNS signs that responded to thiamine; thus, a putative diagnosis of thiamine-responsive polyoencephalomalacia was made. There is a relationship between dietary sulfur and the occurrence of thiamine-responsive polyoencephalomalacia (Gould, 2000). The team concluded that reasonable evidence existed that the emissions may have produced a transient respiratory disease in feeder cattle (chemical pneumonia) and reduced the feeding performance of these animals and extended the time necessary to carry them on feed. The rancher was evacuated from the farm soon after the blowout, and the feeding regimen was disrupted for 3 or 4 days. The owner of a ranch located 2 km east of the well site reported reproductive

failure, loss of weight and condition, and loss of stamina in horses. The team concluded there was no evidence that the reproductive failure in these mares or the loss of weight and condition were related to emissions from the well. However, it is possible that emissions from the well may have had a transient effect on exercise tolerance in these animals. A similar complaint of reduced exercise tolerance was reported from a second farm approximately 3 km northeast of the well site. In this case, an infectious disease was likely occurring concurrent with exposure to the emissions and was probably the cause of reduced exercise tolerance in these horses. Exposure to well emissions may have had a transient contributory effect. A few of the other farms in the surrounding area also reported a transient ocular and nasal irritation in the animals but did not report any reduction in exercise tolerance, condition, or reproductive performance. The investigation team concluded that in several farms up to a 4-km radius from the well site, emissions from the well probably caused transient ocular and respiratory irritation in livestock, primarily horses and cattle. Irritation of sensitive membranes may also have occurred in livestock more distant from the well site, but it was probably not a significant factor. On a farm approximately 8 km from the well site, an outbreak of infectious respiratory disease (pneumonia) and keratoconjunctivitis (pink-eye) occurred. It was not possible to relate these events directly to emissions from the well. However, it is possible that emissions may have played a minor role in increasing the severity of the disease.

Mostrom (1995) and Mostrom and Campbell (1996) investigated the effects of a leak from a sour multiphase pipeline in two herds of cattle. Other investigators have provided epidemiological findings of this event (Waldner *et al.*, 1998). The sour condensate was from a sour gas field, and the sour gas contained approximately 34% H<sub>2</sub>S. The leak was discovered in January, and it is not known for how long the corroded line under a riverbed had been leaking. The condensate primarily contained C<sub>1</sub> to C<sub>9</sub> aliphatics and mono-aromatics (benzene ring). One of the herds was located immediately adjacent to a river where the pipeline break occurred, and it was later moved to a farm approximately 4.5 km from the site. The other herd was located approximately 4 km from the site. The herds were exposed to emissions from the pipeline and also to emissions from the cleanup operations. Burning fugitive sour condensate and washing river gravel were the methods used to remove crude petroleum from river ice, the river bottom, and riparian lands. Emissions from the cleanup operations were freely released into the atmosphere. Cow and calf mortalities occurred. This investigation found clinical evidence of exposure to an irritating substance and evidence of endocrine disruption and immune and nervous system dysfunction. Infections with organisms

considered opportunists (*Enterococcus fecalis* and *Corynebacterium xerosis*) were observed in calves. Some cows developed aggressive behavior, and some had apparent proprioceptive dysfunction. Pregnant cows were observed *in estrus*, and they did not have physiologic signs of prepartum. Many of the calves lacked the suckling instinct, and some cows failed to nurture newborn calves. More than 1400 tissue specimens were collected. Stair *et al.* (1996) reported on the histopathological findings in these herds. Histopathology observed included tracheal hyperplasia of the submucosal glands, discharge of the goblet cells and squamous metaplasia, thyroid and lymphoid hypoplasia, cytoplasmic vacuolization of the gasserian ganglion, and a rare cerebellar neuroblastoma. The epidemiologic study reported that cattle were managed to mitigate exposure to pollution from the sour gas leak (Waldner *et al.*, 1998). Calf mortality increased with cold weather at the time of birthing and with the occurrences of twinning. An association between calf mortality and distance from the pipeline corrosion-induced leak site and wind direction in the Red Deer River Valley was not found. Managing cattle to mitigate exposure to pollution was considered a factor in increasing calf mortality. It is important to note that cold temperatures ( $-16^{\circ}\text{C}$ ) have been reported to cause  $\text{SO}_2$  to induce an increase in metabolism and an increase in nutrient requirements of cattle (Komarnisky, 2003).

## HYDROGEN SULFIDE

Hydrogen sulfide is a constituent of sour gas and other crude petroleum. In sour gas,  $\text{H}_2\text{S}$  can be present at a concentration of less than 1% to greater than 90% (Reiffenstein *et al.*, 1992). Incident-related exposures to  $\text{H}_2\text{S}$  are generally exposures to complex chemical mixtures. The lethality of  $\text{H}_2\text{S}$  is similar across species (Reiffenstein *et al.*, 1992). Studies have shown that wells and associated activities are important sources of  $\text{H}_2\text{S}$  (Burstyn *et al.*, 2007). Hydrogen sulfide is used in relative pure form for heavy water production. It is produced by anaerobic digestion and is present in manure and sewer gas, and it has an offensive odor of rotten eggs. Hydrogen sulfide disassociates in water ( $\text{H}_2\text{S} \leftrightarrow \text{HS}^- + \text{H}^+ \leftrightarrow \text{S}^{2-} + 2\text{H}$ ) to give the hydrosulfide anion ( $\text{HS}^-$ ) and the sulfide anion ( $\text{S}^{2-}$ ), and disassociation is affected by pH and buffering capacity of the solution. At pH 7.4 (physiological pH), approximately 33% exists in the undisassociated form and the rest as the hydrosulfide ion ( $\text{HS}^-$ ). Hydrogen sulfide is also lipid soluble and is soluble in cell membranes. These properties are considered important in the toxicity of  $\text{H}_2\text{S}$ .

## Gasotransmitter

Hydrogen sulfide has been identified as an endogenous gasotransmitter. It is produced endogenously in tissues by cystathionine  $\beta$ -synthase, cystathionine  $\gamma$ -lyase, and/or mercaptopyruvate sulfurtransferase (Gadalla and Snyder, 2010; Liu *et al.*, 2011). These enzymes produce  $\text{H}_2\text{S}$  in micromolar to picomolar concentrations, and this range is considered to be the physiologic levels. As a gasotransmitter,  $\text{H}_2\text{S}$  is vasoregulatory and appears to act in cell signaling in inflammation and in nervous system modulation. Hydrogen sulfide is a modulator of potassium, calcium, and chloride ion channels (Tang *et al.*, 2010). Phase I studies on  $\text{H}_2\text{S}$  in rats have shown that it has potential as a drug in preventing cellular damage associated with ischemic-reperfusion injury (Henderson *et al.*, 2011). This likely has links with modulation of ion channels.

## Effects on metabolism and the endocrine system

Mice exposed to 80ppm  $\text{H}_2\text{S}$  for 5min had an approximately 50% drop in oxygen consumption and an approximately 60% reduction in carbon dioxide output (Blackstone *et al.*, 2005). During 6h of exposure, the metabolic rate dropped by approximately 90% and core body temperature dropped to approximately  $2^{\circ}\text{C}$  above ambient temperature, and oxygen uptake and carbon dioxide output were approximately 10% of pre-exposure value. There was a linear relationship between core body temperature and  $\text{H}_2\text{S}$  concentration. No adverse neurological effects were observed. Mice exposed to 150ppm  $\text{H}_2\text{S}$  for 20min survived exposure to 5% oxygen for 1h and then 3% oxygen for 3.5h without apparent detrimental effects (Blackstone and Roth, 2007). A study in piglets showed that 80ppm  $\text{H}_2\text{S}$  increased metabolism and was a hemodynamic stimulant (Li *et al.*, 2008). Similar observations have been made in 24-kg pigs (Drabek *et al.*, 2011). Brain weights from male rats and mice exposed to 80ppm  $\text{H}_2\text{S}$  were significantly reduced (Dorman *et al.*, 2004). There appears to be both a strain and a sex variation in the sensitivity of rats to  $\text{H}_2\text{S}$ . It is of interest that there are no reports of hypoactivity in mice in these studies (Dorman *et al.*, 2004).

## Reproductive toxicology

Studies have shown that development effects of  $\text{H}_2\text{S}$  can occur. Rats were exposed to 20 or 75ppm  $\text{H}_2\text{S}$  7h/day from gestation day 5 to postpartum day 21, and control groups were included for each exposure level (Skrajny *et al.*, 1992). Serotonin and norepinephrine



levels were assayed in the developing rat cerebellum and frontal cortex. In rats exposed to 75 ppm H<sub>2</sub>S, serotonin levels were increased on days 14 and 21 postnatal in the cerebellum and frontal cortex, and norepinephrine levels were significantly increased on days 7, 14, and 21 postnatal in the cerebellum and on day 21 in the frontal cortex. Exposure to 20 ppm H<sub>2</sub>S significantly increased the levels of serotonin in the frontal cortex on day 21 postnatal, and the levels of norepinephrine were significantly reduced in the frontal cortex on days 14 and 21 and on day 14 in the cerebellum. Exposures to H<sub>2</sub>S at 20 and 50 ppm for 7 h/day from day 5 postcoitus to day 21 postnatal were shown to alter developing cerebellar Purkinje cell dendritic branching (Hannah and Roth, 1991; Roth *et al.*, 1995). In a fertility and developmental study, rats were exposed to 0, 10, 30, or 80 ppm H<sub>2</sub>S 6 h/day, 7 days/week for 2 weeks prior to breeding, during a 2-week mating period (Dorman *et al.*, 2000). Exposures continued from gestation day 0 through gestation day 19. Exposure of dams and pups resumed between postnatal days 5 and 18. No changes in the fertility indices were reported. Male F<sub>0</sub> rats in the 80-ppm group had testicular tubular degeneration, and dose-response linkages to H<sub>2</sub>S exposure were not established. A litter of pups in the 30-ppm exposure group developed epidermolysis bullosa-like lesions. The authors considered this to be the first report of this lesion in the rat, and its linkage to H<sub>2</sub>S exposure is unknown. No other teratologic lesions that can possibly be related to H<sub>2</sub>S exposure were reported. No pathophysiologic neuropathies were reported. The cerebellum was not evaluated by histopathology for arrangement of the Purkinje cell dendrite fields. Sulfide could be an endocrine disruptor by blocking or reducing the number of oxytocin receptors in rat uterus and may modulate uterine contractions (Hayden *et al.*, 1989; Hu *et al.*, 2011). Hydrogen sulfide is also implicated in insulin regulation and in regulation of the hypothalamic-pituitary axis.

## Biochemical toxicology

Hydrogen sulfide disrupts biochemical processes. The biochemical effects of H<sub>2</sub>S have been investigated (Reiffenstein *et al.*, 1992; Khan *et al.*, 1990, 1998). Exposure of rats to H<sub>2</sub>S at 100 ppm for 8 h/day, 5 days/week for 5 weeks reduced the activity of erythrocyte superoxide dismutase (SODe), exposure at 10 and 100 ppm reduced the activity of pulmonary cytochrome *c* oxidase, and there was a trend for brain cytochrome oxidase to be decreased. Rats exposed to various concentrations of H<sub>2</sub>S showed that 10 ppm did not suppress the activities of cytochrome *c* oxidase in lung tissue. Exposure to greater than 500 ppm caused greater than 90% suppression of cytochrome *c*

oxidase and succinate oxidase activity in lung tissue. At 200 and 440 ppm H<sub>2</sub>S exposure levels, pulmonary cytochrome *c* oxidase activity increased at 24 and 48 h postexposure. NADH-cytochrome *c* reductase and succinate reductase were not inhibited by H<sub>2</sub>S *in vivo* and by exposure to sulfide *in vitro*. In H<sub>2</sub>S exposures, mitochondrial swelling is an early histopathologic observation that occurs before cellular necrosis. The *in vitro* effects of sulfide, sulfite, and sulfate on the activities of SODe, catalase, glutathione peroxidase (GSHPX), and glucose-6-phosphate dehydrogenase (G6PDH) were studied in hemolyzates of bovine erythrocytes (Khan *et al.*, 1987). A concentration-dependent inhibition in the activities of these enzymes was observed, but their effects on each enzyme were different. The study showed that SODe and catalase activities were most sensitive to sulfide, followed by sulfite and sulfate. The GSHPX activity was most sensitive to sulfite, followed by sulfide, cysteine, and sulfate. The activity of G6PDH was maximally inhibited by reduced GSH, followed by sulfite, and sulfate and sulfide were inhibitory only at high concentrations. Dialysis of the sulfide- and sulfite-inhibited enzymes resulted in complete or partial reversal of inhibitory effects. These enzymes are important in scavenging free radicals.

## Pathology of hydrogen sulfide

Detailed reports on the experimental pathology of H<sub>2</sub>S and sour gas in cattle do not appear to exist in the scientific literature. Concentration and duration of exposure are important with regard to the toxicological effects of H<sub>2</sub>S (Prior *et al.*, 1988). There are reports on the toxicopathology of H<sub>2</sub>S in other species. Ocular lesions in rats included corneal edema and necrosis, and detachment of corneal epithelial cells has been reported for experimental H<sub>2</sub>S exposures of 56 mg/m<sup>3</sup> (Beasley, 1963; Lopez *et al.*, 1989). Calves continually exposed to H<sub>2</sub>S by inhalation developed corneal opacity (Nordstrom, 1975; Beauchamp *et al.*, 1984). Unfortunately, the corneal pathology was not described in these reports.

Hydrogen sulfide is toxic to the respiratory tract, and the toxic effect may be mediated through inhibition of cytochrome *c* oxidase. Exposure of rats to 560 mg H<sub>2</sub>S/m<sup>3</sup> for 4 h caused exfoliation of respiratory epithelia and olfactory cells, with the exfoliation of the olfactory cells continuing for more than 44 h (Lopez *et al.*, 1988). Lopez *et al.* (1987) also reported that exposure of rats to H<sub>2</sub>S concentrations of 0, 10, 200, and 400 ppm for 4 h increased the cellularity of nasal saline washes by 0, 139, 483, and 817%, respectively. Lopez *et al.* (1988) exposed rats to 0, 14, 280, or 560 mg of H<sub>2</sub>S/m<sup>3</sup>, respectively. Squamous epithelium appears to be resistant to the corrosive effects of H<sub>2</sub>S, and lesions were not observed in these regions of the nasal epithelium.

Lesions of the respiratory and olfactory epithelium were observed in all of the rats exposed to 560 mg H<sub>2</sub>S/m<sup>3</sup>. Cellular necrosis, erosions, and ulcers of the respiratory epithelium were observed. Twenty hours after exposure, eroded or ulcerated areas were covered by a thin layer of basophilic cells, and a neutrophilic exudate was observed in the lamina propria and submucosa. At 44 h after exposure, the respiratory epithelium was covered with pre-ciliated secretory cells, and mitotic figures were observed. Specific lesions were observed in the olfactory epithelium. There were multifocal areas of cellular necrosis, and the lesions were more pronounced at 20 h after exposure. Evidence of healing was not observed 44 h after exposure. Rats exposed to 2317.6 mg of H<sub>2</sub>S/m<sup>3</sup> or rats given intraperitoneal injections of sodium bisulfide died within 3 min (Lopez *et al.*, 1989). The rats that were exposed to H<sub>2</sub>S had massive extravasation of eosinophilic fluid into the bronchoalveolar space. Mature rats and mice were exposed (whole body) to 0, 10, 30, or 80 ppm H<sub>2</sub>S for 6 h/day for at least 90 days (Dorman *et al.*, 2004). Pathology was observed in mice exposed to 80 ppm H<sub>2</sub>S. Infiltration of the nasal epithelium or lamina propria by inflammatory cells, predominantly neutrophils, was observed. The infiltrate was often accompanied by serocellular exudate, epithelial hyperplasia or hypertrophy, squamous metaplasia, erosion or ulceration, cystic epithelial invaginations, fibrosis, hyperkeratosis, or goblet cell hyperplasia. Neuronal loss was observed in the olfactory epithelium of rats and mice exposed to 30 ppm H<sub>2</sub>S. A strain and sex difference appears to exist in rats. Ciliated cells in the nasal passages, trachea, and bronchi are sensitive targets for airborne irritants, including H<sub>2</sub>S, SO<sub>2</sub>, and NO<sub>2</sub>. The ciliated cells are part of the mucociliary escalator that transports mucus and foreign particulate matter from the lower respiratory tract toward the pharynx, where it is swallowed, and from the nasal passages to exit at the nares. The cilia of the columnar epithelium are lost following exposure to irritating airborne substances. Loss of cilia or ciliated cells can alter the defense mechanism of the respiratory tract against particulate substances. The olfactory epithelium is considered a sensitive target in the nasal passages for H<sub>2</sub>S. Lesions of the olfactory epithelium could modify maternal behavior by altering offspring recognition.

Massive pulmonary edema was the pathological finding in humans killed by accidental exposure to H<sub>2</sub>S and in rats experimentally exposed to high concentrations of H<sub>2</sub>S (Burnett *et al.*, 1977; Lopez *et al.*, 1986, 1987, 1988, 1989). Rats exposed to approximately 2317 mg H<sub>2</sub>S/m<sup>3</sup> died within 3 min from pulmonary edema (Lopez *et al.*, 1989). The edema fluid was highly eosinophilic and located in perivascular space and the alveoli. Lethal injections of sodium hydrosulfide did not produce pulmonary lesions. Lopez *et al.* (1986) exposed rats to 0, 56,

and 420 mg of H<sub>2</sub>S/m<sup>3</sup> for 6 h. Mild pulmonary edema was observed in rats immediately after exposure to 56 mg H<sub>2</sub>S/m<sup>3</sup> and in rats that were killed by exposure to 420 mg H<sub>2</sub>S/m<sup>3</sup>. Pulmonary edema was described as primarily perivascular, and edema fluid was rarely observed in the alveoli. Pulmonary edema was not observed in rats exposed to 56 mg/m<sup>3</sup> at 18 and 42 h after exposure. Lymphocytic disruption of the lymph nodes and thymus appeared to increase in severity and frequency in the high-dose group. Because nasal ulcers were observed in all of the rats, activation of the immune system could increase the sensitivity of lymphoid tissue to H<sub>2</sub>S. The cells present in the nasal washes were exfoliated epithelial cells and neutrophils. A 3000% increase in the protein concentrations in bronchoalveolar lavage fluids was observed 1 h postexposure in rats exposed to 400 ppm H<sub>2</sub>S. The increase in protein and leukocytes was considered to be the result of alterations of vascular permeability induced by H<sub>2</sub>S. Dorman *et al.* (2004) observed that exposure of rats and mice to 30 or 80 ppm H<sub>2</sub>S caused bronchiolar epithelial hypertrophy and hyperplasia. The epithelial changes were variably associated with peribronchiolar fibrosis and smooth muscle hypertrophy and often mixed inflammation. Brain weights from the male Sprague–Dawley rats and the male B6C3F1 mice in the 80-ppm exposure group were significantly reduced (Dorman *et al.*, 2004).

### Experimental studies: cattle

Nordstrom (1975) exposed calves to ammonia (NH<sub>3</sub>) and H<sub>2</sub>S gases individually or in combinations. Calves were housed and exposed in uniquely designed exposure chambers. A manure pack was established in each chamber to stabilize a background production of NH<sub>3</sub> at approximately 12.95 ± 5 ppm. Hydrogen sulfide was not identified in the manure gas. Calves were placed in the chambers for a 7-day acclimation period and were exposed to the background level of NH<sub>3</sub> during this interval. The acclimation interval was immediately followed by 7-day exposure to H<sub>2</sub>S. A 7-day observation period completed the experiment. The predominant clinical sign in all treatment groups was ocular irritation as indicated by erythema and excessive lacrimation. Ocular irritation was observed at the background levels of NH<sub>3</sub>. Nasal irritation, based on serous discharge from the nose, was observed at 65 ppm of NH<sub>3</sub>, and the serous discharge was profuse at 150 ppm NH<sub>3</sub>. Dry coughing was also observed, especially at 150 ppm NH<sub>3</sub>. At 150-ppm exposure, the calves kept their eyes closed and had irregular shallow breathing. Based on diminishing clinical signs during the exposure periods, the author concluded that the calves appeared to develop some adaptation to NH<sub>3</sub>. The calves were distressed and

depressed by all exposure levels of  $\text{H}_2\text{S}$ . Signs of distress were restlessness, pawing, head shaking, tail switching, blowing through the nose, licking of the nose, and slobbering with the tongue protruding. Signs of depression were heads lowered and eyes closed. Diarrhea and vomiting were observed in some calves. Breath-holding, panting, dyspnea, shallow breathing, and spasmodic coughing were signs of respiratory distress. Epistaxis was also observed. Neurological effects (nystagmus and depression) were reported. Ocular irritation appeared to be profound, and at 150 ppm  $\text{H}_2\text{S}$  exposure, the effect was described as degenerative. Photophobia and refusal to open the eyelids were observed at the 20-ppm level of exposure. At the high-exposure level, corneal opacity was severe, and the animals were diagnosed as blind. Keratoconjunctivitis and vesicular keratitis were observed. At the end of the exposure interval, rupture of the cornea seemed imminent. The eyes of white-faced calves appeared to be more susceptible to the irritating and inflammatory effects of  $\text{H}_2\text{S}$  than those of calves with pigmented skin around the eyes. Clinical signs of the effects on the upper respiratory tract were more severe in calves exposed to  $\text{NH}_3$  plus  $\text{H}_2\text{S}$ . The severity of epistaxis increased at the exposures to high levels of  $\text{H}_2\text{S}$  plus  $\text{NH}_3$ , and the author considered the effects to be additive or synergistic. The combination of 150 ppm  $\text{H}_2\text{S}$  plus 150 ppm  $\text{NH}_3$  decreased respiration from a pre-exposure rate of 87/min to 63/min. Based on clinical evaluations of the ocular lesions at the 150-ppm  $\text{H}_2\text{S}$  level of exposure, the author concluded that  $\text{NH}_3$  at 150 ppm decreased the effects of exposure to 150 ppm  $\text{H}_2\text{S}$ . Rumen motility was considered decreased in both rate and intensity. Exposure to  $\text{H}_2\text{S}$  plus background  $\text{NH}_3$  decreased feed consumption by 3.5 and 26% for 20- and 150-ppm levels of exposure, respectively. Animals exposed to 20 ppm  $\text{H}_2\text{S}$  tended to recover appetite during the latter part of the exposure interval. During the 7-day postexposure interval, feed consumption increased and was higher than that for the background  $\text{NH}_3$  values. At levels of 20 ppm  $\text{H}_2\text{S}$  plus 65 ppm  $\text{NH}_3$ , appetite depression in the calves was more or less comparable to that of calves exposed to 20 ppm  $\text{H}_2\text{S}$  plus background  $\text{NH}_3$ . Feed consumption in calves exposed to 20 ppm  $\text{H}_2\text{S}$  plus 150 ppm  $\text{NH}_3$  was decreased 6.5%, or essentially double the appetite depression observed in calves exposed to 20 ppm  $\text{H}_2\text{S}$  plus background  $\text{NH}_3$ . Exposure to 150 ppm  $\text{H}_2\text{S}$  plus 150 ppm  $\text{NH}_3$  caused a 32.5% decrease in feed consumption compared to that of the calves exposed to 20 ppm  $\text{H}_2\text{S}$  plus background  $\text{NH}_3$ . These observations suggest that the ratio of  $\text{H}_2\text{S}$  to  $\text{NH}_3$  in the exposure mixture alters the interactive effect. Clinicopathology observations were reported. Sulfhemoglobin was not detected in the blood from calves exposed to 20 ppm  $\text{H}_2\text{S}$ . Statistically significant changes were not observed in levels of blood ammonia, urea nitrogen and uric acid, serum

concentration of bilirubin, glucose, phosphorus, calcium, total protein, albumin and cholesterol, or the activities of the enzymes glutamic oxalacetic transaminase, lactic acid dehydrogenase, and alkaline phosphatase. Leukocytosis was observed in two calves exposed to  $\text{H}_2\text{S}$  plus background concentrations of  $\text{NH}_3$ .

Goats were essentially continuously exposed to  $\text{H}_2\text{S}$  during a 96-h interval in an exposure hood delivery system (Hayes, 1972). The exposure hood controlled the ambient environment around the head and neck of the animals but permitted the animals to eat and drink. Exposure concentrations of  $\text{H}_2\text{S}$  were as follows: 0.0 ppm, four goats; 10 ppm ( $13.9 \text{ mg/m}^3$ ), four goats; 50 ppm, four goats; and 100 ppm, five goats. Goats exposed to 50 or 100 ppm  $\text{H}_2\text{S}$  trembled during activity. A decrease in urinary volume corresponded to a decrease in water consumption. At 50 ppm of exposure, epiphora occurred 24 h after exposure and persisted for the remainder of the exposure interval. Epiphora also occurred with exposure to 100 ppm  $\text{H}_2\text{S}$ , and ocular injury was visible following 24–48 h of exposure. Corneal opacities, which were considered to be reversible, caused partial loss of vision. There was a trend for exposure to  $\text{H}_2\text{S}$  to decrease intake of feed. In animals exposed to 10 ppm, there was a trend to recovery during the exposure period, and the overall decrease in feed consumption was 20%. Exposure to 50 ppm  $\text{H}_2\text{S}$  decreased feed and water intake, especially on the first day of exposure. Although a trend to recovery was observed, complete recovery did not occur. For the 50- and 100-ppm exposure groups, feed consumption decreased on exposure day 2 (37% decrease for the 100-ppm group) and remained depressed through exposure day 4. For the 10-ppm group, there was a decrease in water consumption on exposure day 1 and a rebound on days 2–4. For the 50-ppm group, there was a sharp decrease in water consumption on exposure day 2. For the 100-ppm group, water consumption was also decreased. The author concluded that there was a dramatic decrease in the desire of the goats to consume food and water. Plasma levels of cortisol were increased 48 and 55% in the 50- and 100-ppm groups, respectively. Respiratory and heart rates and blood pressure were recorded each day at 08:00, 10:00, 13:00, and 16:00 h. These data suggest that initial exposure to  $\text{H}_2\text{S}$  at the 10- and 20-ppm levels caused a decrease in respiratory rate on day 1 of exposure. The 0.0- and 10-ppm treatment groups were not different with regard to respiratory rate; exposure to 50 ppm significantly decreased respiration from 19.8 respirations/min on exposure day 2 to 16.5 respirations/min on day 3; goats exposed to 100 ppm had a significant decrease in respiratory rate on the last 2 days of exposure. Exposure to  $\text{H}_2\text{S}$  did not significantly alter heart rate, blood pressure, or rectal temperature; however, there was a trend for rectal temperature to increase during the exposure period.

## EXPOSURE TO ENVIRONMENTAL SULFUR

The impact of elemental sulfur on cattle, especially to producers in close proximity to sulfur stockpiles, is a concern to cattle producers. A study was done on the impact to cattle of fugitive sulfur and other pollution from sulfur mines in Poland (Janowski and Chmielowiec, 1981). The principal pollutants were sulfur dust, sulfur gases, SO<sub>2</sub>, and sulfur hydride. Fifty-five cattle within 1 km of the mine were evaluated during the summer months on pasture and during the winter months when the cattle were predominantly stabled. The control group of 27 cattle was kept in an area that was free of sulfur pollution, 20 km from the mine. Analyses for sulfur dust and sulfur compounds are summarized in Table 61.8. Clinical findings in the polluted area were (1) decreased occurrences of infestation with external parasites and mycotic skin infections (ringworm); (2) increased body temperature and heart and respiratory rates; and (3) decreased nociceptive response. Other findings were abnormal eyes (90.4%), respiratory disease (94.2%), and digestive disorders (21.1%). Clinical findings in the eye were epiphora, erythema of the conjunctiva, and edema of the eyelids. Abnormal findings of the respiratory system included dyspnea, coughing, mucopurulent nasal discharge, and abnormal pulmonary sounds. Digestive disorders included abnormal ruminal motility and diarrhea. Cattle were healthier when kept indoors. Cattle in areas polluted with sulfur, compared to controls, had a decreased burden of parasites in the digestive tract. Pathological findings in the sulfur-exposed cattle were as follows: the respiratory tract contained gray-yellow tinged mucus; inflammation of the trachea; thinning of cellular layers in the bronchi, almost to the point of squamous metaplasia; and interstitial inflammation. The authors concluded that sulfur pollution produces intoxication in the form of a chronic disease that they called sulfurosis.

## TOXICOLOGY OF SULFUR DIOXIDE IN CATTLE

Sulfur dioxide emissions are an important issue with regard to oil and gas pollution because a single sour gas plant removing sulfur from sour gas and operating at efficiencies approaching 100% can have stack emissions of approximately 10 metric tons of SO<sub>2</sub> per day (Waldner, 2001). A relatively small area with several sour gas plants can have emissions of 67–85 tons of SO<sub>2</sub>/day. Inhalation studies (head-only exposure) in cattle showed that SO<sub>2</sub>

TABLE 61.8 Concentrations of sulfur and sulfur compounds in sulfurosis study

Parameter	Study areas	Control areas
Urinary sulfur	Barn, 3.01 g/L Outdoors, 5.33 g/L	0.52 g/L
Milk sulfur	1.87 g/L	0.30 g/L
Serum sulfur	2.58 g/L	0.46 g/L
Sulfur dust	Barn, 1.02 g/m <sup>2</sup> Outdoors, 1.31 g/m <sup>2</sup>	ND
Sulfur dioxide	Barn, 0.089 mg/m <sup>3</sup> (0.034 ppm)	ND
Hydrogen sulfide	Barn, 0.178 mg/m <sup>3</sup> (0.128 ppm) Outdoors, 0.089 mg/m <sup>3</sup> (0.064 ppm)	Barn, ND Outdoors, 0.002 mg/m <sup>3</sup>

ND, not detectable.

had an effect on the immune system and on metabolism, and cold temperature had an effect on SO<sub>2</sub> toxicity (Komarnisky, 2003). A brief summary of this study is given. Eight steers were progressively exposed to room air containing 1 ppm SO<sub>2</sub> for 10 days, 5 ppm SO<sub>2</sub> for 7 days, and 20 ppm for 7 days. Four steers were exposed to SO<sub>2</sub> at room temperature (~18.5°C), and four steers were exposed to SO<sub>2</sub> at -16°C. In the cold environment, the metabolic rate was increased by 33, 39, and 44% at 1, 5, and 20 ppm of SO<sub>2</sub>, respectively. Exposure to SO<sub>2</sub> in the warm environment did not significantly alter metabolic rate. For a 500-kg steer intermittently exposed to 1 ppm of SO<sub>2</sub>, the extra feed required for maintenance and growth would be equivalent to an additional 1.5 kg/day of grain or 2.1 kg/day of hay. Sulfur dioxide was shown to have immunotoxic effects in cattle. Exposure of steers to 5 and 20 ppm in the cold environment decreased the respiratory bursts in neutrophils. Exposure to SO<sub>2</sub> in the cold environment decreased the threshold dose for shedding of respiratory epithelial cells into bronchoalveolar lavage (BAL) fluid. Neutrophil numbers in BAL were increased by exposure to SO<sub>2</sub>, and exposure to cold temperature further increased neutrophil numbers. Exposure to SO<sub>2</sub> decreased the number of pulmonary macrophages in BAL fluid. At the 20-ppm level, lactate dehydrogenase was increased in the BAL fluid.

## PATHOLOGY OF PETROLEUM AND OIL FIELD CHEMICALS

### Clinical pathology

Barber *et al.* (1987) reported on the toxicology of aviation turbine fuel in heifers. Ten animals were considered to



have elevated serum activity of aspartate aminotransferase, interpreted as a reflection of acute hepatic dysfunction, and the activity of  $\gamma$ -glutamyl transpeptidase (GGT) was considered to be within the normal range. Elevated concentrations of nonesterified fatty acids were observed in 11 of the animals, 2 had elevated concentrations of blood urea nitrogen, and 5 had a leukocytosis. Six weeks after the initial farm visit, 41 of 41 animals had elevated serum activity of GGT.

Bystrom (1989) found that sweet crude oil did not alter hematological parameters. There was a correlation between the severity of clinical signs and increased concentrations of plasma fibrinogen. The activity of hepatocellular enzymes in serum and serum glucose was not consistently increased after exposure to sweet crude oil, but serum calcium and potassium values consistently decreased and bilirubin values consistently increased. Rowe (1972) found that hematological parameters, especially leukocyte numbers, were elevated as chemical pneumonia advanced. A single dose of sour crude oil caused a constant decline in plasma glucose. A transient decline in plasma glucose was observed in calves given sweet crude oil or kerosene. Exposure to grease has been reported to cause changes in hematology and clinic chemical parameters (Wallace and Blodgett, 1996). A cow exposed to lithium grease had leukopenia ( $3.7 \times 10^9$  white blood cells/L), lymphopenia ( $1.8 \times 10^9$  lymphocytes/L), and hyperfibrinogenemia (6 g/L, with the normal given as  $<5$  g/L). Triaryl phosphates (TAP) have greater affinity for pseudocholinesterase, also known as plasma cholinesterase or butyrylcholinesterase, than for acetylcholinesterase (Abou-Donia and Gupta, 1994). Erythrocytic acetylcholinesterase was inhibited for a short duration after a calf was initially dosed with 10 g of TAP/kg BW, and the dose was repeated as 5 g/kg BW on day 30 and as 10 g/kg BW on day 56 (Dollahite and Pierce, 1969). In four animals poisoned with tri-*o*-tolyl phosphate, changes in the routine automated clinicochemical and hematological parameters were not observed (Nicholson, 1974). In an experimental study, three calves were given oral doses at 5, 10, or 20 g of TAP/kg BW, with the total dose divided equally over 10 days (Beck *et al.*, 1977). On day 8 or 9, there was a 70–77% reduction in the activity of whole blood acetylcholinesterase. In one calf given 5 g/kg BW as a single oral dose, activity of whole blood acetylcholinesterase was reduced by 68% on day 2 and by 78% on day 9. In cows dosed with 0.5 or 1.0 g of TAP/kg BW, activity of whole blood cholinesterase was reduced by 50 and 63%, respectively, on day 2 and by 56 and 61%, respectively, on day 7. The consensus is that a relationship between the inhibition of blood cholinesterases and organophosphate ester-induced delayed neurotoxicity does not exist (Abou-Donia and Gupta, 1994).

Exposure to nitrates from drilling fluids can cause methemoglobinemia. The concentration of nitrate and nitrite can be determined in rumen fluid, aqueous humor (anterior chamber of the eye), urine, plasma, and serum. Nitrate is reduced to nitrite, and nitrite oxidizes iron in hemoglobin to form methemoglobin.

### Pulmonary pathology of petroleum

The lung is a target for unweathered (unchanged by exposure to atmosphere) petroleum. Hydrocarbon-induced chemical (inhalation) pneumonia occurs when oil is inhaled. Aspiration of oil during emesis is the most common mechanism of inhalation exposure (Coppock *et al.*, 1986b, 1996). Necropsy findings of oil-induced chemical pneumonia have been described (Dungworth, 1985, 1993). Generally, the cranioventral lobes of the lungs are affected first. During necropsy, oil may be identified in the lungs by oil discoloration and a petroleum odor, visible oil in the lung, oil floating to the surface when a piece of lung is immersed in water or formalin, or oil layering on the surface of the supernatant when a piece of lung is homogenized. The pulmonary histopathology for unweathered petroleum is similar for the various forms of petroleum. Histopathological changes are proliferative, and macrophages with a foamy-appearing cytoplasm are found in and tend to fill the alveoli (Dungworth, 1985). In the pathogenesis, oil-containing macrophages are found in the lymphatics, especially those adjacent to blood vessels around bronchi. Fibrosis and proliferation of type II macrophages are prominent features. Foamy macrophages may be incorporated into the alveolar septa by fibroblastic proliferation. As determined by histochemistry and special stains, lipids are found in both intracellular and extracellular sites, and oil can be identified in the alveolar spaces. In the early stages, the lesion may be an acute necrotizing fibrinous bronchopneumonia. Generally, neutrophils, lymphocytes, and plasma cells surround the necrotic areas. Fibrinous exudate, inflammatory cells, and amorphous eosinophilic and oily material can fill the alveolar spaces. Plant material from aspirated ingesta may also be seen in alveoli. Fibrin hemorrhages and a mixed population of mononuclear cells may be observed on the pleural surface. Extensive coagulative necrosis and suppuration may be observed in the consolidated areas. Bacteria may be present in the necrotic areas and alveoli. Hematogenous exposure of the lungs to petroleum hydrocarbons also occurs. Volatile components of petroleum are absorbed by the gastrointestinal tract and are volatilized from the blood in the lungs. Oil emboli can occur in the lungs through absorption of oil from the gastrointestinal tract and form oil emboli that block the small blood vessels in the lungs and brain. Oil emboli

in blood vessels of the lungs and brain have not been reported in cattle intoxicated with crude petroleum. The pathogenesis and observed lesions may be altered by concurrent bacterial infection and cytotoxicity of the oil.

There are reports of pulmonary pathology for field and experimental-induced petroleum intoxication. Ulceration of the ventral aspect of the trachea can occur in kerosene poisoning, and the ulcers may be covered with a pseudomembrane (Rowe, 1972; Rowe *et al.*, 1973). Areas of the lung can be consolidated and have a dark purple, reddened, or tan-gray mottled discoloration. The areas of consolidation often have a nodular appearance. Fibrinous pleural adhesions and serofibrinous pleural exudate can occur. Pulmonary abscesses can also be observed (Rowe, 1972; Rowe *et al.*, 1973). Multiple small hemorrhages were observed in the lungs of cattle poisoned with tractor vaporizing oil (Parker and Williamson, 1951). Chemical pneumonia was reported in cattle dying from diesel fuel poisoning (Gibson and Linzell, 1948). Pneumonia and pulmonary abscesses containing gram-negative organisms were found in cattle that died acutely from drinking water contaminated with aviation turbine engine fuel (Barber *et al.*, 1987). The rumen contents smelled of oil, and the odor matched the source of the petroleum. The carcasses appeared to repel scavengers and flies, and they appeared to have retarded putrefaction. Microscopic examination of tissues from the animals that died in the first 3 weeks showed fatty degeneration of hepatocytes and focal areas of hepatitis, and one of four had tubulonephrosis, renal vascular thrombi, and interstitial nephritis. A diagnosis of pneumonia and abscesses containing gram-negative organisms was made in two of four of the animals. Approximately 124 days after the ingestion of aviation turbine fuel-contaminated water, the remaining cattle were necropsied. At slaughter, the lungs of the cattle were considered abnormal and were characterized by enlargement and gray-blue areas of varying and abnormal firmness. However, histopathological abnormalities were not observed. Infection in the lungs can occur rapidly following chemical injury. Cattle that survive for 6 weeks or longer with chemical pneumonia usually have marked loss of weight and chronic ill health (Rowe *et al.*, 1973).

Pulmonary lesions have been observed in sheep following a 1-day exposure to natural gas condensate-contaminated water (Adler *et al.*, 1992). Gross pathological observations included severe bilateral consolidations and tan to gray mottling of the ventral lobes. Histological diagnosis of the lesions was a necrotizing bronchopneumonia. There were extensive areas of coagulation necrosis bordered by a dense zone of neutrophils and lesser numbers of lymphocytes, plasmacytes, and macrophages. The alveoli were filled with fibrinous exudate and amorphous eosinophilic material.

Plant material was also observed in the pulmonary parenchyma. The pleural surface was coated with fibrin, hemorrhages, and mononuclear cells. Pathogenic and potentially pathogenic bacteria (*Mannheimia (Pasteurella) haemolytica*, *Klebsiella pneumoniae*, *Fusobacterium necrophorum*, *Actinomyces (Corynebacterium) pyogenes*, *Escherichia coli*, and *Pseudomonas* sp.) were isolated from the lungs of some of the sheep.

Experimental studies have been done with crude oil (Bystrom, 1989). Cattle were administered a sweet crude oil per gavage. Many of the animals, especially those that received larger doses of oil, had black discoloration of the lungs. Visible oil was present in the cranio-ventral lobes. In one animal, it was estimated that 80% of the lungs were affected by black discoloration, and visible oil was observed. Pathological diagnoses included pulmonary consolidation, fibronectrotizing pneumonia, pleuritis, emphysema, atelectasis, multifocal interstitial pneumonia, proliferative alveolitis, bronchopneumonia, and alveolitis. In animals that survived 8 days, hyperplasia of type II epithelial cells was observed, and the alveoli were filled with foamy, alveolar macrophages.

Lung lesions can occur when animals are exposed to gaseous petroleum and sour gas. Stair *et al.* (1996) reported lesions of hyperplasia of the submucosal glands in the trachea of cattle exposed to emissions from a multiphase sour gas leak (Mostrom and Campbell, 1996). Other lesions observed included hyperplasia of tracheal, bronchial, and bronchiolar glands; metaplasia of the tracheal mucosa; and loss of cilia from the respiratory epithelium. Lymphoid hypoplasia was also observed. A study was done in feral cats that were exposed to emissions from fires in Kuwait (Moeller *et al.*, 1994). The cats were collected in Kuwait approximately 8 months following ignition of the oil wells. To ensure that the cats had been exposed for the entire duration of the oil field fires, only cats with permanent teeth were examined. Twelve cats were collected in Kuwait City, an area that was relatively smoke-free, and 14 cats were collected in Ahmadi, a city that was partially evacuated because of intense smoke from the fires in the adjacent Bergan oil field. The findings are summarized in Table 61.9. The authors concluded that these lesions were probably reversible and were most likely caused by exposure to pollution from the oil field fires. However, other causes, such as infectious diseases and parasitism, could not be completely eliminated.

## Hepatic pathology of petroleum

Hepatic pathology can be observed following exposure to oil. Parker and Williamson (1951) reported fatty changes in the liver from a cow that had ingested tractor vaporizing oil. Fatty degenerative changes and focal

TABLE 61.9 Respiratory tract lesions observed in feral cats exposed to emissions from the Kuwait oil field fires

Lesion	Kuwait City	Ahmadi <sup>a</sup>
Mild accumulation of anthracotic pigment	5/12	11/14
Hyperplasia of bronchial and bronchiolar glands	3/12	5/14
Scattered occurrence of bronchi that were dilated and filled with cellular debris and a few neutrophils; connective tissue around bronchiolar glands contained lymphocytes and plasma cells	1/12	1/14
Hyperplasia of smooth muscle around terminal and secondary bronchioli	4/12	10/14
Tracheal gland hyperplasia and segmental thickening of the submucosa	3/12	4/14
Multifocal squamous metaplasia of tracheal mucosa	7/12	10/14
Minimal hyperplasia of the submucosal glands of the larynx, some thickening of the laryngeal submucosa, and slight goblet cell hyperplasia	2/12	0/14
Multifocal squamous metaplasia of the laryngeal epithelium	2/12	3/14

<sup>a</sup>Area of intense smoke from oil well fires.

areas of hepatitis were observed in cattle following ingestion of water contaminated with aviation turbine engine fuel (Barber *et al.*, 1987). An abnormal-appearing liver in a heifer was attributed to the ingestion of gasoline 15 months previously (Albert and Ramey, 1964). Histopathology was not provided. Pembina Cardium crude oil was found to be hepatotoxic in cattle (Bystrom, 1989). Pathological changes included swelling of the liver, increased friability, and centrilobular congestion. Cows dosed with crude oil had an increase in liver weight. Histologically, the hepatocytes contained numerous vacuoles, and these vacuoles were thought to have previously contained oil. Wallace and Blodgett (1996) reported the histopathological changes in the liver of a cow that consumed Li grease containing 2050 µg Li/g of grease. The observations were vacuolated and atrophied hepatocytes. Experimental oral exposure of cattle to lithium produced similar hepatic lesions (Johnson *et al.*, 1980). Hepatic changes observed in sheep poisoned by natural gas condensate were fatty degeneration and periportal infiltration of lymphocytes and plasma cells. Biliary hyperplasia and periportal fibrosis were also found (Adler *et al.*, 1992).

### Gastrointestinal pathology of petroleum

In oral exposure, the gastrointestinal tract has primary contact with ingested petroleum. The chemical composition of the oil will cause variation in the pathology observed. Petroleum can be observed and the odor of petroleum can be detected in the rumen and intestinal tract (Wallace and Blodgett, 1996). Kerosene-induced ulcers of the esophagus have been reported (Rowe *et al.*, 1973). Bystrom (1989) reported pyloric ulcers of the abomasum of cattle exposed to unweathered sweet crude oil. She did not report other gastrointestinal lesions. Oil-soaked ingesta can be stiff and sharp because of water repellency and puncture the gastrointestinal tract. Bumstead (1949) reported oil to be very irritating to the

intestinal tract of cattle. Ingestion of natural gas condensate was observed to produce reddening of the serosal surface of the gastrointestinal tract of sheep (Adler *et al.*, 1992). Hemorrhage into lumen of the gut was also observed. Histological observations were submucosal and mucosal congestion and mucosal and serosal hemorrhage. An inflammatory response was observed in all areas of the gastrointestinal tract. Superficial erosions, mild acute cryptitis, and neutrophilic and lymphocytic exudates were reported. Isolated necrosis of enterocytes was also observed. Lithium grease has been reported to cause gastroenteritis in cattle (Johnson *et al.*, 1980).

### Renal pathology of petroleum

The kidneys are also target organs for petroleum. Some animal species and genders within a species are more sensitive to the nephrotoxicity of petroleum than others. The sensitivity of cattle to the nephrotoxicity of petroleum has not been well defined. A cow that consumed Li grease had mild swelling and vacuolation of the proximal tubular cells (Wallace and Blodgett, 1996). Experimental oral exposure of cattle to Li grease containing 1250 µg Li/g of grease produced similar renal lesions (Johnson *et al.*, 1980). Parker and Williamson (1951) reported degeneration of the kidney in cattle after the cattle consumed tractor vaporizing oil. Tubulonephrosis, renal vascular thrombi, and interstitial nephritis were observed in cattle following environmental exposure to aviation turbine engine fuel (Barber *et al.*, 1987). Renal lesions were not observed in surviving animals slaughtered 124 days later. Renal lesions have been observed in cattle dosed with a light crude oil (Bystrom, 1989). These lesions were shrunken or collapsed glomeruli (two to five in a 4 × field), mild focal necrosis of epithelial cells in the collecting ducts, and inflammatory cells that were observed in the renal cortex. In sheep poisoned with natural gas condensate, tubular epithelial necrosis was observed (Adler *et al.*, 1992). Granular eosinophilic

casts (negative for hemoglobin) and protein droplets filled Bowman's space and many renal tubules. Inflammatory cells were seen around tubular casts.

### Nervous system pathology of petroleum

Cattle exposed orally to Li grease had slight hemorrhage into the cerebellar peduncles (Johnson *et al.*, 1980; Wallace and Blodgett, 1996). Lesions in the central nervous system were observed in sheep poisoned by natural gas condensate (Adler *et al.*, 1992). Mild perivascular hemorrhage was seen in the pia mater and in the white matter of the cerebellum and cerebrum. Increased separation between the pia and arachnoid membranes was prominent, and edema was observed in the stroma of the choroid plexus. Lesions of the central nervous system were observed in harbor seals (*Phoca vitulina*) exposed to crude petroleum (Prudhoe Bay crude oil) from the T/V *Exxon Valdez*, which grounded in Prince William Sound (Spraker *et al.*, 1994). Lesions were observed in neurons and axons of the midbrain. Histopathological observations included intramyelinic edema of the large myelinated axons, neuronal swelling, neuronal necrosis, and axonal swelling and degeneration of the midbrain. These lesions were most prominent in the thalamic nuclei. Lesions of the central nervous system were also observed in polar bears. The bears were dermally exposed to Midale crude oil (Oritsland *et al.*, 1981). The lesions consisted of degenerated and necrotic cells in the small cell pyramidal layer of the hippocampus. Other lesions observed were perineuronal vacuolation in the cerebral cortex. Enlargement of the Virchow–Robin spaces and extravasation of blood around small blood vessels occurred.

### Cardiac pathology of petroleum

Cardiac lesions associated with petroleum poisoning in cattle do not appear to have been reported, but they have been observed in sheep exposed to petroleum condensate (Adler *et al.*, 1992). Gross pathological changes included epicardial hemorrhages, serosanguineous pericardial fluid, pale-appearing myocardium, and reddened endocardium. Microscopic observations were segmental myocardial necrosis and calcification. The myofibrils had loss of cross-striations and a beaded appearance. There were multifocal areas infiltrated with lymphocytes, macrophages, and occasional neutrophils. The blood vessels were congested.

### Pathological effects of petroleum in embryos

Lesions in the bovine fetus linked to exposure to crude oil have not been reported. However, studies on the

embryotoxicity of Prudhoe Bay crude oil (PBCO) have been performed. Couillard and Leighton (1989, 1990a,b) reported that PBCO was embryotoxic and that the toxicity was different from hypoxia induced by sealing the eggs with a sealing wax. Mineral oil (USP), used as a control, was not toxic to chicken embryos. Dosages ranged from 2 to 20  $\mu$ L PBCO/egg; the oil was applied on the surface of the egg below the airspace. Hepatic lesions were hepatocellular necrosis, mineralization, and perivascular and multifocal accumulations of heterophils. Hepatocellular necrosis was dose dependent. The liver was observed to have superficial yellowish zones that were multifocal to diffuse. These yellowish zones and mineralization corresponded to areas of hepatocellular necrosis. Mineralization occurred in the necrotic areas. Vacuolation of the hepatocytes was observed. The number of mitotic figures in the liver increased with the dose. Renal lesions were limited to the mesonephros. The capillaries of the glomeruli were distended, and cellular casts and mineralization were observed in the renal tubules. Splenic lesions were limited to increased granulopoietic cells arranged in distinct cords. Other pathological findings were ascites and subcutaneous edema. Hepatic lesions were observed 2 days after treatment; renal and splenic lesions were observed on day 3. Edema fluid was more pronounced after day 3.

In another study, the toxicity of PBCO was evaluated in chicken embryos (Lusimbo and Leighton, 1996). The chicken embryos were exposed to oil on day 9 of incubation. The oil was placed on the eggshell over a prominent blood vessel of the chorioallantoic membrane. Dosages ranged from 0 to 16  $\mu$ L PBCO/egg, and embryonal mortality was dose dependent. Exposure to PBCO at 4  $\mu$ L decreased weight gain during a 3-week posthatching observation period. Lesions were observed in the liver, subcutaneous tissues, bursa of Fabricius, and pipping muscle (musculus complexus). Hepatic lesions were multifocal to locally extensive areas of hepatic necrosis and mineralization. Large fluid-filled vesicles were observed on the dorsocaudal aspect of 5 and 16% of embryos dosed with 1 and 2  $\mu$ L of PBCO, respectively. The bursa of Fabricius had depleted lymphoid tissue, and the interstitium was infiltrated with heterophils. Lesions in the pipping muscle were edema and hemorrhage, sparse multifocal fragmentation, and occasional vacuolation of the myofibers.

### Sour gas and sour condensate

Stair *et al.* (1996) reported on cattle that were exposed to sour gas/condensate from a break in a multiphase pipeline. Pathological findings in the respiratory tract included hyperplasia of the submucosal tracheal glands, discharged goblet cells, squamous metaplasia of the



tracheal mucosa, and submucosal perivascular lymphoid infiltration. Also observed was hypoplasia of the lymph nodes. These effects were associated with exposure to leaked sour gas/condensate in an area of intensive sour gas and crude oil production and processing.

### Nonpesticide organophosphorus esters

Nonpesticide phosphate esters are used as additives for lubricating oils operating under extreme conditions. Coppock *et al.* (1995a) published a review of the neurotoxicity of these esters. The pathology of TAPs has been described in several species (Craig and Barth, 1999). Specific pathological changes may not be observed during necropsy (Dollahite and Pierce, 1969; Nicholson, 1974; Beck *et al.*, 1977). Irritation and hemorrhage in the gut have been associated with TAP poisoning (Julian *et al.*, 1976). Histopathological changes of axonopathy may progress in severity toward the cell body (Maydew *et al.*, 1983; Jubb and Huxtable, 1992). The peripheral axonal lesion was described as demyelination and swelling ( $3\times$  to  $4\times$ ) of the axonal cylinders (Dollahite and Pierce, 1969). Vacuolation and degeneration of axons were observed in the sciatic and femoral nerves (Julian *et al.*, 1976). Dollahite and Pierce (1969) observed demyelination in the spinal nerve roots, of which the ventral nerve roots were the most severely affected. Cytoplasmic vacuolation of the large motor neurons was also observed. Lesions of the spinal cord were described by Nicholson (1974) as axonal degeneration in the gray matter of the ventral horn and in the ascending nerve tracts. Swelling of the axonal cylinder ( $20\times$ ), with a transition from basophilic to acidophilic staining properties, was observed in the spinal cord of cattle inadvertently or experimentally exposed to TAPs (Beck *et al.*, 1977). From lumbar to cervical regions, the lesions appeared to change in pattern and severity. Histological evidence of axonal degeneration was not observed in the L5 vertebral region; however, a few swollen axons were observed at the level of L2 and posterior thoracic (T12) vertebrae. A marked increase in degenerated axons appeared at the level of T5 and the seventh cervical vertebrae (C7). At the level of C3, the lesions were more severe at the tips of the dorsal and lateral horns. Axonal degeneration was not observed in the brain. Lesions of demyelination and degeneration were observed bilaterally in the ascending tracts of the cervical regions of the spinal cord (Julian *et al.*, 1976). The lesions were immediately ventral to the dorsolateral sulcus in the area of the spinocerebellar tract and in the fasciculus gracilis. The degenerating areas had large vacuoles containing eosinophilic globules. Prantner and Sosalla (1993) reported multifocal areas of spongiosis in the medial and lateral funiculi at the levels of vertebrae L2–L5. Focal perivascular hemorrhages

were also observed in the basal nuclei of the brain. The chemical composition of the TAP, age of the animal, and the ascending progression of lesions when they were observed may explain these discrepancies (Gupta and Abou-Donia, 1994).

### Sulfur

Pathological findings in environmental sulfur poisoning of cattle were gray-yellow tinged mucus in the respiratory tract, inflammation of the trachea, thinning of cells in the bronchus almost to the point of squamous metaplasia, and interstitial inflammation (Janowski and Chmielewicz, 1981). Sulfur dioxide produced histopathological evidence of severe irritation of the respiratory tract and eyes. Manure gas poisoning produced congestion and edema of the lungs, gastroenteritis, and peritoneal effusions.

## EPIDEMIOLOGICAL STUDIES: CATTLE

Scott and colleagues used licensed  $\text{SO}_2$  emissions from sour gas plants to estimate the exposure of cattle to emissions from oil and gas fields (Scott *et al.*, 2003a–d). Using Alberta Agriculture extension survey data for beef cattle, these authors found that there was no association for annual risk of culling, calf crop delivered, stillbirth, twinning, mortality, and calf crop weaned and exposure to  $\text{SO}_2$ . For dairy herds using Dairy Herd Improvement data, there was no association with time to culling and no association for annual culling risks and mortality. There was a small risk for increased calving interval with high exposure levels. Geographic area was interactive, suggesting the effect of emissions is subject to modification by soil type, vegetation, and climate.

A study was done in the Caroline–Sundre area of Alberta (Waldner *et al.*, 2001a,b). Seven beef herds were non-randomly selected for study, and herds with health problems previously identified with oil and gas field pollution were not included in the study. Associations were identified for exposure to unaudited industry reported oil and gas field pollution and increased calving interval, increased calf mortality, decreased cow productivity, and increased risk of stillbirth. Lead dioxide-impregnated gauze in Petri plates and filter paper impregnated with zinc acetate were used as static monitors to measure  $\text{SO}_2$  and  $\text{H}_2\text{S}$ , respectively. These static monitors were assayed every 30 days for sulfate and sulfide. Total sulfation increased the risk of odds of non-pregnancy and increased the risk of twinning. Increased  $\text{H}_2\text{S}$  increased the calving-to-calving interval and

increased the risk of nonpregnancy and abortion. The relationship between non-audited industrial reported emissions and total sulfation was not reported.

A large epidemiologic study was done on the effects of oil and gas pollution in beef cattle (WISSA, 2006; Waldner, 2008a–c). Static monitors were used to determine predominantly monthly averages of selected emissions from oil and gas field operations, including sour gas plants, gas gathering plants, and batteries (Burstyn *et al.*, 2007). Assessing exposure parameters for pollutants that are complex chemical mixtures over a wide area is difficult. The authors stated that one uncertainty in the results may be selections of split points. A substantial portion of the variability in analytical values occurred in sampling and analytical techniques. Distance from the wells rapidly decreases the concentrations of H<sub>2</sub>S, and the number of sour gas wells increases the expected concentration of H<sub>2</sub>S. Including flaring and sourness (sulfur content) of the wells improved the model fit. The primary predictor of SO<sub>2</sub> was the distance of the sampler to gas processing plants. The results suggest that SO<sub>2</sub> levels peaked during December to March. There was poor correlation between the logarithms of H<sub>2</sub>S and SO<sub>2</sub>, suggesting that SO<sub>2</sub> levels are not a good predictor for H<sub>2</sub>S levels. Sampling equipment was operating close to the lower range of sensitivity.

The positive associations with exposure to oil and gas field pollutants that have been reported are given. Calving interval in mature cows exposed to greater than 0.236 µg benzene/m<sup>3</sup> was estimated to increase by 3 days compared to that of mature cows exposed to less than 0.236 µg benzene/m<sup>3</sup> (Waldner and Stryhn, 2008). Exposure to SO<sub>2</sub> at the time of calving also increased the risk of calf mortality (Waldner, 2008d). Exposure to SO<sub>2</sub>, H<sub>2</sub>S, and volatile organic compounds measured as benzene or toluene and also SO<sub>2</sub> in the first month after calving, was associated with a minor increased likelihood of calf treatment after the first month of life. There was a linear relationship between H<sub>2</sub>S exposure and the risk of treatment. The proximity to sour gas flares was not examined. In calves that died, increasing postnatal exposures to benzene and toluene were associated with increased odds of respiratory lesions in calves older than 3 weeks (Waldner and Clark, 2009). Increasing prenatal exposure to SO<sub>2</sub> increased the odds of lesions in skeletal muscle or myocardium. The odds were higher in calves exposed to levels greater than 9 ppb SO<sub>2</sub>. Thyroid lesions of several types, including hypoplasia and lack of colloid, necrosis and degeneration, and hyperplasia, were recorded in calves aborted, stillborn, or dying postnatally. Changes were most common in stillborn calves and least in calves born alive. Calves with thyroid lesions often had other pathology as well. More work is needed to determine the importance of these findings (Waldner *et al.*, 2010).

Immune parameters in yearling cattle from 22 herds were examined for effects of ambient PM<sub>1.0</sub> and selected polyaromatic hydrocarbons (PAHs). Immune parameters were peripherally circulating B lymphocytes and CD4, CD8, γδ, and WC1 T-lymphocyte subtypes (Bechtel *et al.*, 2009a). The antibody response to rabies vaccine was also evaluated. No associations between PM<sub>1.0</sub> and selected PAHs were demonstrated in the epidemiologic models employed. In another model, in which exposures to SO<sub>2</sub>, H<sub>2</sub>S, benzene, and toluene were examined, exposure to toluene was associated with significant CD4 T lymphocytopenia (Bechtel *et al.*, 2009b). The number of CD4 T lymphocytes was 30% lower in cattle exposed to 0.823 µg/m<sup>3</sup> toluene than in cattle exposed to less than 0.406 µg/m<sup>3</sup> toluene. In a similar model, 360 newborn calves (24 h to 7 days of age) were studied (Bechtel *et al.*, 2009c). The exposure period examined was from breeding date to birth. The circulating CD4 and CD8 T lymphocyte numbers were 42 and 43% lower, respectively, in calves exposed to 0.378 µg/m<sup>3</sup> or greater of benzene compared with calves exposed to less than 0.276 µg/m<sup>3</sup> benzene. Also, CD4 T lymphocytes were 40% lower in calves exposed to 0.713 µg/m<sup>3</sup> or greater of toluene compared to calves exposed to less than 0.348 µg/m<sup>3</sup> toluene.

## WILDLIFE

The estimated production of crude oil in 2007 was approximately  $3.1 \times 10^{10}$  barrels, and approximately 50% of the oil produced was transported in international shipping lanes (Rogowska and Namiesnik, 2010). When marine spills occur, all marine animals can be at risk of intoxication and hypothermia. Birds and marine mammals are at risk of losing insulation and buoyancy after feathers or fur have been oiled. A study has shown that feathers behave as a wick, and exposure to oil has a cumulative impact on feathers (O'Hara and Morandin, 2010). It is estimated that subvisible oil sheen can reduce the insulation properties of feathers. Oil also affects metabolism in birds, and overcompensation to cold occurs (McEwan and Koelink, 1973). Birds exposed to crude oil from the "Prestige" spill were examined by pathologic methods (Balseiro *et al.*, 2005). Birds examined were predominantly common murrelets (*Uria aalge*), razorbills (*Alca torda*), and puffins (*Fratercula arctica*). Dead and live birds were classified: group 1 ( $n = 5715$ , 29%) were found dead on beaches and had varying degrees of coverage with oil, group 2 ( $n = 5172$ , 7%) comprised dead birds from the beaches that were not covered by oil, and group 3 ( $n = 51578$ , 64%) birds were recovered alive from beaches and had varying levels of oil coverage. Tissues examined by histopathology

were lung, liver, kidney, spleen, ventriculus, proventriculus, intestine, and uropygial gland. Birds in group 1 were observed to have 10–100% oil coverage and to be emaciated with severe atrophy of pectoral muscles and remarkable absence of body fat. Hemosiderin was observed in the Kupffer cells and macrophages in spleen and kidney. Urates were observed in liver, spleen, and kidney. Birds in group 2 had evidence of diarrhea, and they had hemorrhages in lungs, liver, spleen, intestine, and kidney. Birds in group 3 had previously been cleaned. They were dehydrated and had emaciation and aspergillosis of lung, air sacs, kidney, and liver. Severe dehydration and emaciation is a common finding in marine birds that have been soiled with oil. Crude petroleum in food (0.05 mL crude oil/g food/day) decreased egg production in domestic ducks (*Anas platyrhynchos*) (Harvey *et al.*, 1981). Eggshell thinning was observed. The short-day-induced decrease in serum prolactin was more pronounced in the oil-treated birds, and they were slower in restoring prolactin when exposed to long-day photoperiod. Plasma levels of corticosterone were decreased in the oil-treated birds. Plasma thyroxine ( $T_4$ ) levels were decreased in the oil-treated birds, and triiodothyronine ( $T_3$ ) levels were not changed.

In another study, south Louisiana crude petroleum in food (0.03 mL crude oil/g food/day) produced an 84% decrease in the daily rate of oviposition and increased atretic follicles (Holmes *et al.*, 1978). There was a trend for eggshell thinning. In a study in mallard ducks, dietary south Louisiana crude oil (0.03 mL crude oil/g food/day) caused a decrease in plasma estradiol during the ovulatory cycle (Cavanaugh and Holmes, 1982). In a similarly designed study, crude oil adversely altered corticosterone endocrine functions (Gorsline and Holmes, 1982). Dietary petroleum increased cold stress mortality in saltwater-adapted birds (Holmes *et al.*, 1979). The deaths were attributed to changes in adrenal gland function. A likely mechanism is altered metabolism of steroid hormones in the adrenal gland (Coppock, 2011; Coppock and Dziwenka, 2011). Thyroid activity in nestling tree swallows (*Tachycineta bicolor*) was examined in wetlands partially filled with mine tailings from exploitation of oil sands (Gentes *et al.*, 2007). Compared to control nestlings,  $T_4$  levels in the thyroid gland and plasma levels of  $T_3$  were elevated in nestlings from polluted sites compared to those from the reference site. These findings suggest that endocrine disruption occurred. In a companion study, nestling growth and hepatic ethoxyresorufin-*o*-deethylase (EROD) and nesting success were evaluated (Gentes *et al.*, 2006). Harsh weather was devastating to chicks (48% mortality) on control sites, and polluted sites had 59–100% chick mortality; the odds of chick mortality in the polluted site were 10 times higher. In the subsequent year, with a less harsh weather, chick weights at the polluted sites were less.

The hepatic activity of EROD of chicks on the polluted sites was increased. Xenobiotic-linked up-regulation of hepatic enzymes could be linked to the changes observed in thyroid hormones and could alter steroid metabolism (Coppock, 2011).

## ANALYTICAL TOXICOLOGY

Various analytical techniques can be used to match petroleum oil in the lungs and gastrointestinal tract of an animal with the source of the petroleum. Oil on feathers and fur can also be matched by analytical methods with the source. Petroleum gas in water wells can be matched to the oil- and gas-producing formations in the area. The best procedure is to contact the laboratory and request instructions for appropriate sampling containers and methods of preservation. Care must be taken that lubricants on clippers and other devices do not contaminate the samples. The sampling procedure must be written out, signed, and dated. Labels must correspond with the description of the sample. Pictures should be taken and a log made of the photographs. Glass containers should be used, and the containers must be chemically clean. The majority of samples can be stored by freezing. Care must be taken to ensure that samples do not break during freezing and that the frost on the container during thawing does not alter the label. Plastic containers have the disadvantages that substances may migrate into the sample and they present expensive cleanup problems for the analytical laboratory. If aluminum foil is used to seal containers, the dull side has contact with the sample. Pictures should be taken and a description of the pictures written. The tissues that should be collected at necropsy appropriate for the anatomy of the species include rumen and stomach contents, contents from various areas of the gastrointestinal tract, liver, kidney, lung (including description of the lobe), perirenal or bone marrow fat, brain, and soiled hair or feathers. Samples should be taken of the suspect petroleum. Environmental samples include soil, water with floating substances, and water plus sediment. The suspect water and oil should be sampled. The analytical chemistry is complex because petroleum and the compounds associated with petroleum and oil and gas field operations are complex mixtures of chemicals. The most common approach is to match chromatographic fingerprints. The chromatographs are extracts from tissues such as lung and other tissues and chromatographs of the suspect petroleum. Chemicals in petroleum that are resistant to biodegradation are called biomarker chemicals (e.g., phytane and pristane). Many of the biomarkers in crude petroleum are sensitive to petroleum refining

and upgrading processes. Some biomarkers can have some level of accumulation in fat. The percentage of fat found in tissue extracts must be recorded along with the concentration of the biomarker of interest. If possible, multiple biomarkers should be used and the ratio of the biomarkers calculated for the suspect petroleum and the biomarkers found in tissue. For flare emission issues, analyses of tissues, especially fat, may show the presence of a number of PAHs. However, there are generally multiple sources of these PAHs. In the assay of produced water, ions and heavy metal pollutants can be assayed. In addition to the concentration of the ions or metals, the ratios of the ions and metals can be helpful in identification and tracing the production water to its source. For example, if production water has contaminated domestic water, the ratio of the ions in the domestic water should reflect the ion ratio found in the produced water.

## DIFFERENTIAL DIAGNOSIS

The diagnosis of petroleum toxicity can be straightforward or very difficult, depending on the circumstances. If the exposure is current or recent and there is direct evidence of toxic substances in or on the animals and obvious detrimental effects, the diagnosis may still require careful assessment and testing, but it should be accomplished relatively easily. However, if the exposure has not been recent and clinical signs are not specific or are ones that may have several possible causes, the diagnosis can be very difficult. Pneumonic lesions associated with crude oil toxicity are easier to interpret if oil is still present in the gastrointestinal tract. The presence of vacuolated macrophages is helpful in differentiation from infectious causes of pneumonia. The anatomic site of injury from toxic gases depends on the dose, chemical reactivity, aqueous solubility, and particle size. Water-soluble gases such as  $\text{SO}_2$  usually cause upper respiratory injury. Less soluble gases may cause alveolar injury (Caswell and Williams, 2007). Pneumonia initiated by toxic gases is difficult to diagnose because the initial site of injury is at the bronchoalveolar junction, the same location as the initial site of infection for several viral and bacterial pathogens. The bronchiolitis and alveolar reactions of necrosis, edema, and cellular infiltration can be indistinguishable between toxic gas responses and infectious agent responses. In addition, toxic gases can interfere with the normal functions of cells lining the trachea and bronchi, leading to reduced resistance to infection (Dungworth, 1993). Multiple agents may also be present at the same time, making the determination of the initial cause impossible. Acute neurological signs

may result from sodium ion toxicity. The differential diagnosis includes thiamine deficiency-linked polioencephalomalacia, which results in brain lesions that must be distinguished from those of lead poisoning. The analysis for sodium levels in rumen content is essential. The occurrence of delayed neurotoxicity signs requires an in-depth investigation of potential causes, including TAPs and organophosphorus pesticides, to determine possible exposure. Poor growth and weight loss may have many possible causes, including nutritional deficiency, parasitism, chronic pneumonia, chronic intestinal disease, and mycotoxicosis. Temporal connection to petroleum sources such as crude oil and previous acute cases is important in the differentiation. The possible role of adverse effects of petroleum compounds on the immune system in increasing susceptibility to infectious disease further complicates the situation. Reproductive losses may have multiple causes. The time delay often present between exposure and clinical signs makes diagnosis very difficult, if not impossible. Abortions that result during an acute toxic episode are the easiest to associate with the toxin. The potential hormonal disruption from chemicals that disturb the endocrine hormone system relating to conception and pregnancy may not be possible to determine days or weeks after the exposure.

Signs of irritation of the eyes and respiratory tract may be observed after exposure to irritating gas. The occurrence of ocular and respiratory irritation in the presence of toxic gases is a typical sign. However, similar irritation may result from dry, dusty, and windy conditions and from irritating particles in feed. The measurement of toxic gas levels in the vicinity of the livestock is seldom possible, and assumptions of exposure based on data from the emission source and nearby monitoring sites may be the only data available. Isolating the infectious agent and ruling out toxic gas exposure may make differentiation from infectious pinkeye.

## CONCLUSIONS AND FUTURE DIRECTIONS

Domestic and wild animals ingest petroleum and chemicals used in the exploration and production of oil and gas. Many of the chemicals used have limited toxicology information, and the toxicology of chemical mixtures is unknown. The toxicology in aquatic organisms may be known because of the requirement for toxicology assessments before use on offshore platforms.

Areas that require future research are analytical methods and toxicological assessment in laboratory exposure systems. Definitive toxicology data are required before epidemiological studies can be accurately interpreted.



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# Polychlorinated biphenyls, polybrominated biphenyls, polychlorinated dibenzo-*p*-dioxins, and polychlorinated dibenzofurans

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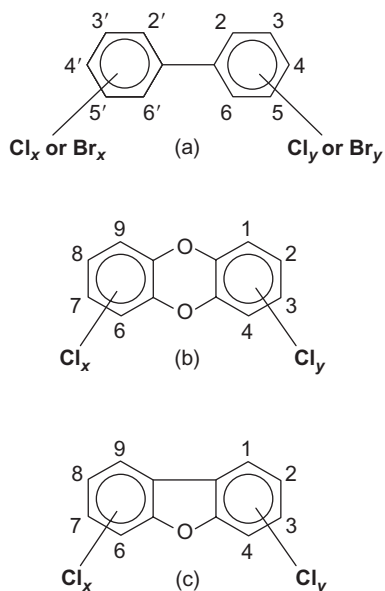
## INTRODUCTION

Polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) form a large group of compounds, the polyhalogenated aromatic hydrocarbons (PHAHs), that are structurally related and are environmentally and biologically persistent (Safe, 1990, 1998; Van den Berg *et al.*, 1994; Fries, 1995; Huwe, 2002; Mandal, 2005; Schecter *et al.*, 2006).

PCBs and PBBs were produced commercially for a variety of applications, whereas the PCDDs and PCDFs occur as byproducts of industrial and natural processes. The structurally similar PCBs and PBBs are formed by substituting chlorine or bromine, respectively, for hydrogen on the biphenyl molecule that consists of two benzene rings (Figure 62.1). Theoretically, there are 209 possible PCB and PBB congeners considering the five chlorine or bromine binding sites on each ring. Each congener is assigned a unique number from 1 to 209 in accordance with the rules of the International Union of Pure and Applied Chemistry (IUPAC). Commercial PCB and PBB products were mixtures of congeners that differed with respect to the extent and positions of chlorination or bromination. PCDDs are composed of two benzene rings connected by two oxygen bridges and contain four to eight chlorines, for a total of 75 congeners (Figure 62.1). PCDFs are also composed of two

benzene rings that are fused to a furan ring in the middle. The benzene rings each have four chlorine binding sites (Figure 62.1). There are 135 different PCDF congeners (DiCarlo *et al.*, 1978; Safe, 1990, 1998; Fries, 1995; Headrick *et al.*, 1999; Huwe, 2002; Mandal, 2005; Schecter *et al.*, 2006; Erickson and Kaley, 2011).

Certain approximate stereoisomers in this group, often referred to collectively as dioxins and dioxin-like compounds (DLCs), induce a common suite of effects and have a common mechanism of action mediated by binding of the PHAH ligand to a specific high-affinity cellular protein. This group of dioxins and DLCs includes 7 PCDD congeners, 10 PCDF congeners, and 12 PCB congeners. Although the PBB congeners analogous to the 12 PCB congeners could also be considered dioxin-like chemicals, the relatively short commercial life span and restricted environmental distribution of PBBs generally preclude them from consideration. The prototype for the dioxins is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicity and persistence of the PHAHs are determined by structure, with lateral substitutions on the ring resulting in the highest degree of toxicity. For the PCDDs and PCDFs, congeners with chlorines in the 2, 3, 7, and 8 positions fall into this category. The TCDD-like PCB congeners are the non-*ortho*-substituted compounds with no chlorines on the 2, 2', 6, or 6' position and mono-*ortho*-substituted compounds with no more than one chlorine on the 2, 2', 6, or 6' position (Safe, 1990; Fries, 1995; Headrick *et al.*, 1999; Huwe, 2002; Mandal, 2005; Schecter *et al.*, 2006).



**FIGURE 62.1** Structures and numbering of generic PCB/PBB (top), PCDD (middle), and PCDF molecules (bottom).

Mechanistic studies indicate that the toxic and biochemical effects associated with exposure to TCDD and related PCDDs, PCDFs, and PCBs are mediated by initial binding of the chemical to the cytosolic aryl hydrocarbon receptor (AhR) present in target tissues and organs. There is a correlation between the AhR binding affinity of these chemicals and their structure-toxicity relationships, which supports the idea that the AhR is involved in the mediation of responses induced by TCDD and related PCDD, PCDF, and PCB congeners (Okey *et al.*, 1994; Hahn, 1998, 2002; Safe, 1998; Denison *et al.*, 2002; Denison and Nagy, 2003; Mandal, 2005).

The common mechanism of action of TCDD and related compounds allows for use of the toxic equivalency factor (TEF) approach to estimate the TCDD-like toxicity of complex mixtures containing chemicals that resemble TCDD. The TEF for a TCDD-like congener is a number that reflects the potency of the individual congener relative to TCDD. Using the TEF concept, the total TCDD toxic equivalents (TEQs) for a complex mixture containing TCDD-like chemicals can be calculated to provide an estimation of the total TCDD-like toxicity. Toxic equivalents contributed by each TCDD-like chemical in the mixture are determined by multiplying the concentration of the congener by its TEF (Safe, 1998; Huwe, 2002).

The PCDDs, PCDFs, and PCBs are widely distributed into the global environment due to long-range transport, and they can be very resistant to environmental degradation and metabolism. As a result, they readily accumulate in the food chain, with the greatest tissue concentrations being found in species at the higher

trophic levels. Residues have been detected in a variety of animal species, including humans (Van den Berg *et al.*, 1994; Safe, 1998). In some situations, the environmental concentrations of these contaminants are such that there is a health risk to animals and humans. Because of this risk, there continues to be an effort on the part of state, federal, and international regulatory agencies to minimize exposure to this significant class of environmental contaminants.

## BACKGROUND

### Sources of PCBs, PCDDs, PCDFs, and PBBs

#### PCBs

PCBs were initially synthesized by Schmidt and Schultz in 1881 and were first manufactured commercially in 1929 by the Swann Chemical Company in Anniston, Alabama. In 1935, the Monsanto Company, based in St. Louis, Missouri, purchased the Swann Chemical Company and continued production of Swann's line of polychlorinated polyphenyl products marketed under the trade name of Aroclor. This product line included the widely known PCBs as well as polychlorinated terphenyls (PCTs) and mixtures and blends of PCBs and PCTs. Most PCBs were known as liquid Aroclors, and PCTs and the most highly chlorinated PCBs were known as solid Aroclors. In naming the liquid PCB products, the trade name Aroclor was followed by a four-digit number in which the first two digits were "12," which designated the product as a refined PCB, and the last two digits referred to the average percentage of chlorine, by weight, in the product. For example, Aroclor 1260 was a refined PCB product containing 60% chlorine by weight. Monsanto was responsible for most of the U.S. production of PCBs, which continued until 1977 (Erickson and Kaley, 2011). Similar PCB mixtures were produced by other manufacturers worldwide through the 1980s. Trade names of these products included Clophen (Bayer, Germany), Pheoclor and Pyralene (Prodelec, France), Fenchlor (Caffro, Italy), Kanechlor (Kanegafuchi, Japan), Chlorfen (Poland), Sovol (the former USSR), and Delor (Chemko, the former Czechoslovakia) (Kimbrough, 1987, 1995; Safe, 1994; Erickson and Kaley, 2011).

There were three major use categories of PCBs: completely closed systems (electrical equipment including capacitors and transformers), nominally closed systems (hydraulic and heat transfer systems and vacuum pumps), and open-ended applications (plasticizer in polyvinyl chloride, neoprene, and other chlorinated rubber). PCBs were also components of surface coatings, paints, inks, adhesives, and pesticide extenders. In

addition, they were used for microencapsulation of dyes for carbonless copy paper, immersion oils for microscopes, catalysts in the chemical industry, and as cutting oils and lubricating oils. Capacitors and transformers were the primary uses of PCBs (approximately 50 and 25%, respectively), whereas hydraulic and lubricating fluids accounted for approximately 6% (Safe, 1990; Headrick *et al.*, 1999; Erickson and Kaley, 2011).

The physical and chemical properties of PCBs, such as high stability, inertness, and dielectric properties, that were advantageous for many industrial purposes led to the international use of PCBs in large quantities (Tanabe, 1988). For example, the estimated cumulative production of PCBs in the United States between 1930 and 1975 was 700,000 tons, and an estimated 1.2 million tons were produced worldwide. Domestic sales of PCBs in the United States during this time period totaled 627,000 tons (Kimbrough, 1987, 1995; Tanabe, 1988).

PCBs were identified in environmental media and biota as early as the late 1960s. Some uses of PCBs resulted in environmental releases, such as the recycling of PCB-containing carbonless copy paper. However, the majority of PCBs were sealed in electrical equipment and thus only entered the environment as the result of accidents, maintenance, or disposal after years of service (Erickson and Kaley, 2011). By early 1970, Monsanto started to address the presence of PCBs in the environment, and in 1971 the company voluntarily withdrew PCBs from all markets that were considered likely to lead to environmental discharges. Sales were restricted to a limited number of manufacturers of electrical equipment (capacitors and transformers), which was considered a closed-end use. In 1977, 2 years before the U.S. Environmental Protection Agency's (EPA) ban of the manufacture of PCBs, Monsanto ceased production entirely (Kimbrough, 1987, 1995; Erickson and Kaley, 2011).

Although PCBs are no longer used commercially because of their persistence, they are still present in the environment. Approximately 31% (370,000 tons) of all the PCBs produced is present in the global environment. It is estimated that 780,000 tons are still in use in older electric equipment and other products, deposited in landfills and dumps, or in storage (Tanabe, 1988).

### PCDDs and PCDFs

PCDDs and PCDFs are byproducts that are formed during the synthesis of certain industrial halogenated aromatics chemicals, byproducts from other commercial processes, and byproducts of combustion (Safe, 1990). Some of the important industrial sources of PCDDs and PCDFs have included their formation as byproducts in the production of PCBs, chlorinated phenols and chlorinated phenol-derived chemicals, hexachlorobenzene, technical hexachlorocyclohexanes, and chlorides of

iron, aluminum, and copper. PCDDs and PCDFs have also been identified in effluents, wastes, and pulp samples from the pulp and paper industry and in finished paper products. Emissions from municipal and hazardous waste incinerators as well as home heating systems that use wood and coal, diesel engines, forest and grass fires, and agricultural and backyard burning contain PCDDs and PCDFs. Another contribution might come from naturally formed PCDDs and PCDFs, which have been detected in deep soils and clays from the southern United States and Germany (Safe, 1990; Huwe, 2002).

Historical data suggest that the majority of anthropogenic PCDDs and PCDFs began entering the environment in the 1930s and 1940s, with releases peaking in the 1970s. Since the 1970s, emissions have been decreasing (Bhavsar *et al.*, 2008). EPA estimated that annual emissions of PCDDs and PCDFs decreased from 13.5 to 2.8 kg TEQ/year between 1987 and 1995. This was due primarily to improvements in incinerator performance and removal of incinerators that could not meet emission standards. Other regulations, including bans or restrictions on the production and use of chemicals such as the wood preservative pentachlorophenol (PCP), the phaseout of leaded gasoline that contained halogenated additives, and the elimination of chlorine bleaching in the pulp industry, also contributed to reducing concentrations of PCDDs and PCDFs (Huwe, 2002). There has been a further 50% decline in emissions between 1995 and 2000 from known sources in the United States (Bhavsar *et al.*, 2008).

### PBBs

PBBs were manufactured for use as flame retardants in industrial and consumer products (Damstra *et al.*, 1982). It is estimated that approximately 13 million pounds were produced in the United States from 1970 to 1976 and used by more than 130 companies for incorporation into plastic products that included business machine housings, radios, televisions, thermostats, electric shavers, hand tools, and various automotive parts (DiCarlo *et al.*, 1978; Headrick *et al.*, 1999). Three commercial PBB products were manufactured in the United States: hexabromobiphenyl, octabromobiphenyl, and decabromobiphenyl (DiCarlo *et al.*, 1978; Hardy, 2000). Hexabromobiphenyl was the predominant product, with approximately 11.8 million pounds being produced solely by Michigan Chemical Company (DiCarlo *et al.*, 1978). More than 98% of the hexabromobiphenyl was produced as FireMaster BP-6, with the remainder being produced as FireMaster FF-1 (Hesse and Powers, 1978) after addition of an anti-caking agent to FireMaster BP-6. Michigan Chemical Company stopped production of its PBB products in 1974 (DiCarlo *et al.*, 1978). White Chemical Company and Hexcel Corporation



manufactured octa- and decabromobiphenyl in the United States until 1979 (IARC, 1986). Production of decabromobiphenyl was discontinued in Great Britain in 1977, and Germany stopped production of brominated biphenyls in 1985. In 2000, France discontinued the remaining commercial production of PBBs (Hardy, 2000).

### Environmental fate of PCBs, PCDDs, PCDFs, and PBBs

The release of PCBs into the environment primarily has been the result of leaks, spills, and improper disposal. As stated previously, it is estimated that approximately 370,000 tons of PCBs are present in the global environment (Tanabe, 1988). The volatility of PCBs allows their evaporation from water surfaces and movement through the atmosphere, resulting in widespread environmental dispersal (Headrick *et al.*, 1999).

PCDDs and PCDFs are released into the atmosphere primarily by combustion sources and by evaporation from PCDD/PCDF-containing soils and water. Similar to PCBs, the PCDDs and PCDFs can be transported long distances by winds, contributing both to general background concentrations and to contamination of remote areas far from the original source.

PCBs, PCDDs, and PCDFs are removed from the atmosphere by physical processes such as wet and dry deposition and vapor uptake and are deposited on soils, surface waters, and plant surfaces. Most of the PCBs, PCDDs, and PCDFs that are deposited on surface waters sorb onto suspended sediments. Once bound to soil and sediment, these chemicals generally remain fixed, except for bulk transport due to soil erosion, flooding, and dredging (Dickson and Buzik, 1993). Ingestion of these compounds by animals results in their preferential bioaccumulation and biomagnification in higher trophic levels of the food chain (Safe, 1994).

Because PBBs were manufactured for a relatively short time and because of their restricted use, they are not considered to be a significant environmental contaminant with the exception of specific locations in Michigan related to production and disposal. It is important to remember that the commercial PCB products and PCBs, PCDDs, and PCDFs in environmental extracts are complex mixtures of congeners. Because of various physical and biological processes, the compositions of the commercial PCB mixture and an environmental PCB/PCDD/PCDF mixture may vary significantly from one another. Thus, the impacts of PCBs, PCDDs, and PCDFs on the environment and biota are due to the individual components of these mixtures and their additive and/or non-additive (synergistic/antagonistic) interactions with themselves and other classes of pollutants (Safe, 1994).

### Exposure to PCBs, PCDDs, PCDFs, and PBBs

There are a number of ways by which animals can be and have been exposed to PCBs, PCDDs/PCDFs, and PBBs. Some of the scenarios described involve ingestion of low concentrations of these chemicals through consumption of environmentally contaminated feed or feed components, whereas other scenarios involve accidental incorporation of the chemical into the feed, resulting in exposure to relatively high concentrations of the contaminant. Usually, food animal exposures to PCBs, PCDDs, and PCDFs occur below concentrations resulting in acute toxicity. Clinical signs are not evident, and often there is not a noticeable economic impact on the health of the animal, although there may be detectable contamination of food products such as milk, meat, and eggs (Headrick *et al.*, 1999).

#### PCBs

During the 1940s and 1950s, silos constructed with concrete were sealed with a PCB-containing paint, which eventually peeled off from the walls, resulting in contaminated silage. Dairy and beef cattle were exposed to the paint in the feed, resulting in accumulation of PCBs in adipose tissue. As a result, food products such as milk and meat contained detectable concentrations of PCBs. Examples of other exposure incidents resulting in PCB residues in food animals were summarized by Headrick *et al.* (1999).

#### Michigan PBB incident

The most extensive exposure of food animals and humans to PBBs occurred in Michigan in the mid-1970s. In 1970, the Michigan Chemical Company began to manufacture PBBs under the trade name of FireMaster BP-6 in St. Louis, Michigan. In 1972, the company changed the formulation of the flame retardant and renamed it FireMaster FF-1 (Fries, 1985). In May 1973, 650 pounds of FF-1 was mistakenly included in a shipment of feed-grade magnesium oxide to a feed mill in Climax, Michigan. A portion of the magnesium oxide that was shipped to the feed mill was used to mix feeds primarily for dairy cattle at that location, which were subsequently shipped to area farms or other retail units. The remaining magnesium oxide was shipped to other mills within the state and used in feeds mixed at those locations. Feeds that were not formulated to contain magnesium oxide also became contaminated because of carryover from the contaminated feed-mixing equipment (Fries, 1985). In total, 101 feed mills were affected (Duncel, 1975).

Most of the high-level exposures occurred during the fall of 1973 before sale of the initial batch of feed was

stopped in December 1973 because of dairy producer complaints of animal health problems. Low-level contamination of feed continued beyond the chance identification of PBBs as the contaminant in April 1974 because of their persistence. Because of the long half-life of PBBs, the decision was made to depopulate affected farms and to dispose of the animals at a burial site in northern Michigan. Initially, 9400 head of cattle, 2000 swine, 400 sheep, and 2 million chickens were buried in addition to 865 tons of feed and animal byproducts such as cheese, butter, dry milk products, and eggs. Later, an additional 20,000 head of cattle as well as 3900 swine and 1100 sheep were buried (DiCarlo *et al.*, 1978; Damstra *et al.*, 1982; Fries, 1985; Headrick *et al.*, 1999). In total, 507 farms were affected (Dunckel, 1975).

### PCDDs and PCDFs

For livestock, atmospheric deposition of PCDDs and PCDFs onto forage and soils is assumed to be the major source of exposure (Huwe, 2002). Extensive field studies in contaminated areas have demonstrated a positive correlation between PCDD and PCDF concentrations in animals and their soil contact (Van den Berg *et al.*, 1994). The relative importance of soil depends on the species of animal and the management system. Ruminants are expected to be more vulnerable to PCDD and PCDF exposure than are poultry and swine because of their grazing activities. The use of pasture is of particular importance because consumption of contaminated plants is additive to soil ingestion of PCDDs and PCDFs. Volatilization of PCDDs and PCDFs from soil and deposition on plants is an important pathway of animal exposure when forage is abundant. However, that soil may be more important when grazing is sparse. The soil ingestion pathway is not limited to grazing animals. Cattle confined to unpaved lots consume small amounts of soil that can lead to product residues. Tanabe and Minh (2010) reported that open dumps in developing Asian countries are a potential source of PCBs, PCDDs, and PCDFs. A variety of municipal wastes are disposed of at these sites, where spontaneous low-temperature burning and/or intentionally generated fire are common, resulting in the formation of PCDDs and PCDFs. In addition, PCBs can be leached from old electrical equipment dumped there. Humans residing near the dump sites are at risk from direct exposure to these chemicals, but in some countries, such as India, an additional route of exposure is through consumption of contaminated milk from buffalos and cows that graze on the garbage in the dumps. Although most poultry and pork production is conducted in confined operations, the soil ingestion pathway of exposure may be important when these species have access to contaminated soil (Fries, 1995). Van Overmeire *et al.*

(2009a,b) and Waegneers *et al.* (2009) reported that home-produced eggs in Belgium contained elevated concentrations of PCBs, PCDDs, and PCDFs, which was attributed to consumption of soil and soil organisms by laying hens.

In addition to exposure to PCDDs and PCDFs through consumption of contaminated soil and/or forage, a number of other incidents of animal exposure to these compounds have been reported. The first known exposure to PCDDs occurred in the late 1950s. "Chick edema disease," as the condition was initially called, resulted from consumption of feed containing fat contaminated with a number of PCDD congeners originating from the production of PCP. In 1975, several horses died in Missouri as a result of exposure to TCDD-contaminated waste oil that was used for dust control on horse tracks. In a geographical survey, high concentrations of TEQs in cattle were strongly correlated to PCP-treated wood used for fence posts and feed bunks at several beef cattle operations across 13 states in the United States. Although the use of PCP was restricted in the 1980s, PCP was used heavily on farms as a wood preservative in the late 1970s (Huwe, 2002). In an EPA survey of poultry, samples were found with PCDD concentrations considerably above background. The origin of this contamination was ball clay, which had been added as an anti-caking agent to soy meal in the feed. This same contaminated feed was also used by the catfish industry and resulted in high TEQ concentrations in catfish from Arkansas (Huwe, 2002).

Several dioxin contamination incidents have occurred in Europe. In 1998, during routine monitoring, dairy products were identified that had dioxin concentrations two to four times higher than background concentrations. The source of the contamination was traced to citrus pulp used as a cattle feed component. In another incident, PCB/PCDD/PCDF-contaminated oil was added to recycled fat used as an additive in animal feeds. The affected feeds contaminated Belgian poultry, dairy, and meat and were discovered only after toxic effects characteristic of "chick edema disease" were seen in chickens. Animals and products were quarantined, recalled, and eventually destroyed. The incident led to international recalls and bans of Belgian products (Van Larebeke *et al.*, 2001; Bernard *et al.*, 2002; Huwe, 2002; Guitart *et al.*, 2010). In November 2008, routine sampling of fat from slaughtered pigs in Ireland indicated the presence of non-TCDD-like marker PCB congeners. The source of the contamination was traced to dried bread that had been used as feed. The contaminated dried bread was traced back to a food recycling plant that had used recycled mineral oil as a fuel in direct flame drying of food waste during the processing of animal feed (Casey *et al.*, 2010).

## Differential toxicity

Comparison of the relative toxicities of PCDDs, PCDFs, and PCBs as three separate classes suggests that the dioxins are more toxic than the furans, which in turn are more toxic than the PCBs. It was concluded that PBBs are slightly more toxic than their chlorinated counterparts (McConnell, 1985). Studies also suggest that the toxicity of commercial PCB formulations increases with increasing chlorine content (Aroclor 1221, 1232, 1242, 1248, 1254), but highly chlorinated Aroclors 1260, 1262, and 1268 are less toxic than Aroclor 1254 (Tanabe, 1988).

TCDD binds with the greatest affinity to the AhR and is the most potent congener in terms of toxicity. PCDDs and PCDFs substituted with chlorines in at least three of the four lateral positions (2,3,7,8; Figure 62.1) bind most strongly to the AhR. If chlorines are removed from these lateral positions or if chlorines are added to the nonlateral positions (1,4,6,9; Figure 62.1), binding affinities decrease markedly, as do toxicities. There are seven 2,3,7,8-substituted PCDDs (2,3,7,8-TCDD; 1,2,3,7,8-pentaCDD; 1,2,3,4,7,8-hexaCDD; 1,2,3,6,7,8-hexaCDD; 1,2,3,7,8,9-hexaCDD; and 1,2,3,4,6,7,8-heptaCDD and -octaCDD) and ten 2,3,7,8-substituted PCDFs (2,3,7,8-TCDF; 1,2,3,7,8-pentaCDF; 2,3,4,7,8-pentaCDF; 1,2,3,4,7,8-hexaCDF; 1,2,3,6,7,8-hexaCDF; 1,2,3,7,8,9-hexaCDF; 2,3,4,6,7,8-hexaCDF; 1,2,3,4,6,7,8-heptaCDF; and 1,2,3,4,6,7,8-heptaCDF and -octaCDF) that induce TCDD-like toxicity. There are 209 theoretically possible PCB congeners having different toxic and biologic responses. The most toxic PCB congeners have four or more chlorine atoms at both the *para* (4,4') and the *meta* positions (3,3',5,5'; Figure 62.1) in the biphenyl rings, but they have no chlorine (bromine) atoms in the *ortho* positions (2,2',6,6'; Figure 62.1). Of the 209 PCB congeners, four (3,3',4,4'-tetraCB (IUPAC 77); 3,4,4',5-tetraCB (IUPAC 81); 3,3',4,4',5-pentaCB (IUPAC 126); and 3,3',4,4',5,5'-hexaCB (IUPAC 169)) are approximate stereoisomers of the highly toxic TCDD and thus bind to the AhR and elicit toxic and biologic responses typical of TCDD, although at higher doses. These 4 congeners are considered to be coplanar because both rings of the biphenyl molecule lie in the same plane, which enables binding to the AhR (Tanabe, 1988). There are 8 PCB congeners with chlorine substitution in one of the *ortho* positions (2,2',6,6'). These congeners may have partial coplanarity and thus exhibit lower competitive binding affinities for the AhR and lower toxic potency. The mono-*ortho* PCB congeners are 2,3,3',4,4'-pentaCB (IUPAC 105); 2,3,4,4',5-pentaCB (IUPAC 114); 2,3',4,4',5-pentaCB (IUPAC 118); 2',3,4,4',5-pentaCB (IUPAC 123); 2,3,3',4,4',5-hexaCB (IUPAC 156); 2,3,3',4,4',5-hexaCB (IUPAC 157); 2,3',4,4',5,5'-hexaCB (IUPAC 167); and 2,3,3',4,4',5,5'-heptaCB (IUPAC 189) (Poland and Knutson, 1982; Tanabe, 1988; Safe, 1990, 1998; Giesy and Kannan, 1998; Huwe, 2002; Whyte *et al.*, 2004).

## TEFs

The relationship between the structure of individual PCDD, PCDF, and PCB congeners and their toxicity is the basis of TEFs and the TEQ approach. The TEQ approach is used to determine the toxic potency of complex mixtures of PCDDs, PCDFs, and PCBs found in the environment. Assuming a similar mechanism of action (binding to the AhR), the potency of each chemical in a mixture to cause a particular toxic or biological effect can be expressed as a fraction of the potency of TCDD to cause the same effect. Thus, the TEF is a ratio of  $EC_{50}$  (TCDD-like chemical)/ $EC_{50}$  (TCDD). TCDD has been assigned a TEF value of 1.0. Based on a variety of biological endpoints, relative potency factors (RPFs) are assigned to the different TCDD-like congeners. All RPFs for an individual congener are evaluated to derive a consensus value (the TEF) that describes an order-of-magnitude potency for that congener. The toxic potency of a mixture of PCDDs, PCDFs, and/or PCBs is estimated by multiplying the concentrations of individual congeners by their respective TEFs and summing the products to yield total TEQs. The total TEQs express the toxicity as if the mixture were pure TCDD (Safe, 1990, 1998; Dickson and Buzik, 1993; Van den Berg *et al.*, 1994, 2006; Fries, 1995; Whyte *et al.*, 2004; Schecter *et al.*, 2006). Several assumptions are made when using the TEF approach: (1) the effects of individual PCDDs, PCDFs, and/or PCBs in a mixture are additive; (2) only tissue and environmentally persistent organochlorine compounds have been assigned TEFs; and (3) all of these compounds bind to the AhR and elicit receptor-mediated biochemical and toxic responses (Safe, 1998). The TEQ approach and current values (Table 62.1) have been adopted internationally as the most appropriate way to estimate the potential health risk of mixtures of TCDD-like chemicals (Schecter *et al.*, 2006).

There are some limitations with the TEF approach. Blankenship *et al.* (2008) note that TEFs are consensus values of relative potencies of the various PCB/PCDD/PCDF congeners rather than precise values. They may vary depending on species, measurement endpoint, and the relative proportions of individual congeners in complex mixtures. The assumption that toxic responses to TCDD-like chemicals are additive and that other classes of contaminants do not modify or add to the toxicity clearly is not always correct (Safe, 1998). In addition, some of the mammalian TEFs are based on *in vitro* studies that do not account for whole animal exposure characteristics, including absorption, distribution, metabolism, and elimination, or on quantitative structure-activity relationships because of a lack of toxicity data for some congeners, which can introduce an additional degree of uncertainty into the determination of TEQs. Another limitation of the TEF approach is that it does not consider potential adverse effects of *ortho*-substituted PCB



TABLE 62.1 Summary of World Health Organization (WHO) 2005 toxic equivalency factor (TEF) values

Compound	WHO 2005 TEF
<i>Chlorinated dibenzo-p-dioxins</i>	
2,3,7,8-TCDD	1
1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0003
<i>Chlorinated dibenzofurans</i>	
2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDF	0.03
2,3,4,7,8-PeCDF	0.3
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,6,7,8,9-HpCDF	0.01
OCDF	0.0003
<i>Non-ortho-substituted PCBs</i>	
3,3',4,4'-tetraCB (PCB 77)	0.0001
3,4,4',5-tetraCB (PCB 81)	0.0003
3,3',4,4',5-pentaCB (PCB 126)	0.1
3,3',4,4',5,5'-hexaCB (PCB 169)	0.03
<i>Mono-ortho-substituted PCBs</i>	
2,3,3',4,4'-pentaCB (PCB 105)	0.00003
2,3,4,4',5-pentaCB (PCB 114)	0.00003
2,3',4,4',5-pentaCB (PCB 118)	0.00003
2',3,4,4',5-pentaCB (PCB 123)	0.00003
2,3,3',4,4',5-hexaCB (PCB 156)	0.00003
2,3,3',4,4',5'-hexaCB (PCB 157)	0.00003
2,3',4,4',5,5'-hexaCB (PCB 167)	0.00003
2,3,3',4,4',5,5'-heptaCB (PCB 189)	0.00003

From Van den Berg *et al.* (2006).

congeners. *Ortho*-substituted PCBs have been shown to elicit a variety of non-AhR-mediated toxic responses in experimental animals, including neurobehavioral, neurotoxic, carcinogenic, and endocrine changes. However, studies reviewed by Giesy and Kannan (2002) suggest that it is unlikely that the effects of *ortho*-substituted PCBs are critical to survival at concentrations typically associated with the AhR-mediated toxic effects of PCBs.

## TOXICOKINETICS

### PCBs and PBBs

Because commercial PCB and PBB products are mixtures of individual congeners that differ in the number and position of chlorine or bromine atoms and thus differ in terms of their biological activities, it is difficult to accurately assess their absorption, distribution, metabolism,

and elimination. A number of experiments have been conducted with a variety of species, including cows, pigs, rats, and birds, on the absorption, distribution, metabolism, and elimination of the commercial PBB mixture FireMaster BP-6, and these were summarized in an extensive review by Fries (1985). Because of the similarities between PCBs and PBBs, information pertaining to one can generally be applied to the other.

In general, PBBs are rapidly and extensively absorbed, with absorption being inversely dependent on the number of bromine atoms (Damstra *et al.*, 1982; Fries, 1985). For example, less than 10% of a dose of C<sup>14</sup>-labeled 2,2',4,4',5,5'-hexabromobiphenyl was eliminated in rats (Matthews *et al.*, 1977; Fries, 1985), indicating almost complete absorption, compared to 62% fecal elimination of C<sup>14</sup>-labeled octobromobiphenyl 24 h after dosing, suggesting incomplete absorption (Norris *et al.*, 1975; Fries, 1985).

PBBs are widely distributed throughout the body of all species studied. Initial concentrations are generally greatest in the liver and adipose tissue (Damstra *et al.*, 1982; Fries, 1985), with highest equilibrium concentrations on a wet tissue basis being in adipose tissue (Miceli and Marks, 1981; Damstra *et al.*, 1982; Fries, 1985). Concentrations of PBBs in muscle and organ tissues are usually an order of magnitude lower than those in adipose tissue. Generally, differences in concentration between tissues can be attributed, at least in part, to variations in fat content of the tissues (Fries, 1985).

Individual PBB congeners in FireMaster BP-6 undergo hydroxylation by metabolic routes that are similar for the related PCBs, with the rate of metabolism being determined primarily by the position of bromine atoms on the ring and secondarily by the bromine content of the molecule (Damstra *et al.*, 1982). *In vivo* studies suggest that, as for PCBs, metabolism can occur if there are two adjacent unbrominated positions (Matthews *et al.*, 1978; Fries, 1985).

Elimination of individual PBB congeners occurs at different rates, with congeners that are more slowly removed becoming more concentrated in tissues relative to congeners that are more rapidly eliminated. The predominant congener in FireMaster BP-6, 2,2',4,4',5,5'-hexabromobiphenyl is the most persistent congener in the various species studied (Damstra *et al.*, 1982). PBBs are eliminated primarily by biliary excretion into the feces, but fecal concentrations are low compared to whole-body concentrations (Damstra *et al.*, 1982; Fries, 1985). Placental transfer of PBBs occurs to some extent, but the concentrations in fetal or offspring tissues are relatively small compared to concentrations in maternal tissues. Transfer of PBBs to the offspring during nursing results in much greater whole-body concentrations compared to PBB transfer during gestation. For example, pigs from sows that were fed PBBs during gestation and lactation had a fivefold increase in body burden during



the 4-week lactation period, with residues accumulated during lactation accounting for 95% of the total body burden (Werner and Sleight, 1981; Fries, 1985).

As suggested previously, milk is the major route of elimination of PBBs for lactating mammals, although in the case of females nursing their young, the contaminant is simply transferred from one animal to another. Concentrations of PBBs in milk fat generally exceed dietary concentrations, with bovine milk fat concentrations exceeding dietary concentrations by three- or four-fold (Fries and Marrow, 1975; Willett and Irving, 1976; Damstra *et al.*, 1982; Fries, 1985).

The egg is a major route of elimination for egg-laying birds, with concentrations in chicken or Japanese quail eggs being 1 to 1.5 times that of dietary concentrations (Babish *et al.*, 1975; Fries *et al.*, 1976; Polin and Ringer, 1978a; Damstra *et al.*, 1982; Fries, 1985). Elimination of PBBs via the egg can account for as much as 50% of the daily dose if there is no deleterious effect on egg production (Fries, 1985).

PBBs can have a relatively long biological half-life in animals. Studies with cows suggested biphasic elimination of PBBs via the milk, with an initial half-life of 11 days and a second half-life of 58 days (Fries and Marrow, 1975; Gutenmann and Lisk, 1975; Fries *et al.*, 1976; DiCarlo *et al.*, 1978). In cases in which observation periods were long, a biological half-life of 180 days was estimated for lactating cows (Fries, 1985). It was estimated that the concentration of FireMaster BP-6 in bovine milk fat would decrease from approximately 300 to 0.3ppm in 120 weeks (Detering *et al.*, 1975; DiCarlo *et al.*, 1978). A half-life of 17 days was calculated for FireMaster FF-1 in the eggs of chickens fed a diet containing the commercial mixture. It was estimated that a chicken exposed to 1ppm PBB in the feed for at least 10 days (the minimum time required to attain a steady-state concentration in the contents of a whole egg) would require 87 days of feeding uncontaminated feed to reach a concentration of 0.05ppm in the whole egg (Ringer and Polin, 1977).

## PCDDs and PCDFs

The absorption, distribution, metabolism, and elimination of PCDDs and PCDFs have been extensively reviewed by Van den Berg *et al.* (1994). Absorption from the gastrointestinal tract of mammals is effective and can exceed 75% of the dose for the lower chlorinated congeners. With increasing molecular size, absorption from the intestines is greatly reduced, which is most apparent for the hepta- and octachlorinated congeners. The liver and adipose tissue are the major storage sites of PCDDs and PCDFs for most mammalian and avian species. The biotransformation of PCDDs and PCDFs depends on the

number and position of the chlorine atoms on the molecule. Metabolic reactions include oxidation and reductive dechlorination as well as breakage of the oxygen bonds. Sulfur-containing metabolites have also been identified. In general, the urinary and biliary elimination of 2,3,7,8-substituted congeners depends on the metabolism of these compounds. Whole-body half-lives of the group of 2,3,7,8-substituted congeners in rodents range from a few to more than 100 days.

The absorption of PCDDs and PCDFs from the gastrointestinal tract has been studied for a number of individual congeners. The extent of absorption of 2,3,7,8-TCDD or related compounds is variable, depending on the vehicle and the substitution pattern of the congener. There appear to be no differences between species in terms of absorption of these compounds through the gastrointestinal tract. There are indications that passage across the intestinal wall is predominantly limited by molecular size and solubility, with the influence of these two parameters being most significant for hepta- and octachlorinated congeners, which exhibit decreased absorption. Studies with rats, mice, hamsters, guinea pigs, cows, and chickens in general indicate that 2,3,7,8-tetra- and pentachlorinated congeners are well absorbed from the gastrointestinal tract (50–90%), and octachlorodibenzo-*p*-dioxin is absorbed only to a limited extent (2–15%) (Pohjanvirta and Tuomisto, 1994; Van den Berg *et al.*, 1994).

The tissue distribution of PCDDs and PCDFs has been extensively studied in laboratory experiments using rodents and nonhuman primates. The liver and adipose tissue are the major storage sites of PCDDs and PCDFs for most mammalian and avian species, whereas depending on species, the skin and adrenals can also act as primary sites for deposition. Several studies with rats, mice, hamsters, guinea pigs, and monkeys indicated that the 2,3,7,8-substituted PCDDs and PCDFs are the predominant congeners retained in tissues and body fluids. Studies suggest that the tissue distribution of TCDD and related compounds is dose dependent in that as the dose increases, so does the liver:adipose distribution ratio. In the liver, TCDD induces both cytochromes CYP1A1 and CYP1A2. Induced CYP1A2, in turn, appears to be a crucial binding species for TCDD and related compounds in rodents. The hepatocellular binding of TCDD to CYP1A1 is so strong that only a very limited amount will be released back into the circulation. Placental transfer of 2,3,7,8-substituted PCDDs and PCDFs was found to be strongly dependent on molecular size. In a number of mammalian species, the transfer of PCDDs and PCDFs from the mother to the offspring via lactation is quantitatively more important than transport to the fetus across the placenta. Excretion via lactation generally decreases as chlorine content increases (Pohjanvirta and Tuomisto, 1994; Van den Berg *et al.*, 1994).

As in mammals, the liver and adipose tissue of avian species are the major sites for storage and accumulation of 2,3,7,8-substituted PCDDs and PCDFs. Hepatic deposition of 2,3,7,8-substituted PCDDs and PCDFs appeared to increase with increasing chlorination, resulting in a limited transfer of the more highly chlorinated congeners to the egg (Van den Berg *et al.*, 1994).

Metabolism of TCDD and related compounds is necessary for urinary and biliary elimination, thus playing a major role in regulating the rate of excretion of these compounds. In rats, metabolic reactions include oxidation, preferably in the lateral positions, and reductive dechlorination as well as oxygen bridge cleavage of the diphenyl ether in 2,3,7,8-substituted PCDDs. Metabolism of PCDDs and PCDFs is generally thought to be a detoxification process; thus, toxicity is attributable to the unchanged parent compound (Pohjanvirta and Tuomisto, 1994).

The induction of CYP1A1 and CYP1A2 enzyme activities by 2,3,7,8-substituted PCDDs and PCDFs has been shown to be one of the most sensitive parameters for biological activity of these compounds. The enzymes most studied are the CYP1A1-dependent ethoxyresorufin-*ortho*-deethylase and aryl hydrocarbon hydroxylase. In addition, 2,3,7,8-substituted PCDDs and PCDFs also induce phase II enzymes (Van den Berg *et al.*, 1994).

In mammals, the liver and adipose tissue are the major compartments for the deposition of PCDDs and PCDFs. The elimination of polar metabolites of 2,3,7,8-substituted PCDDs and PCDFs occurs predominantly via the bile and feces, with urinary excretion playing a minor role. However, urinary elimination plays an important role in the hamster. In rats, the whole-body half-lives of PCDDs and PCDFs range from 17 to 31 days, depending on the dose and strain of rat used, whereas in hamsters and mice, the whole-body half-lives range from 11 to 15 and from 11 to 24 days, respectively. In rats, it was shown that lactation can reduce the half-life of these compounds, whereas egg-laying can reduce the half-life in avian species. In lactating cows, mean half-lives ranged from 40 to 50 days for tetra- to heptaCDDs and CDFs. 2,3,7,8-Tetrachlorodibenzofuran (TCDF) is eliminated more rapidly than TCDD, having a whole-body half-life of 2 days. The rapid elimination of TCDF is thought to be due to its rapid metabolism. In contrast, 2,3,4,7,8-pentaCDF (PeCDF) is eliminated very slowly in the rat, with a whole-body half-life of 64 days. The slow elimination rate is probably due to tight binding of this congener by CYP1A2, in addition to limited metabolism. As chlorine content increases, the rate of elimination of PCDDs and PCDFs decreases (Pohjanvirta and Tuomisto, 1994; Van den Berg *et al.*, 1994).

Interspecies differences in toxicity can only be partly explained by differences in toxicokinetics. The hamster is the species most resistant to the acute toxicity of TCDD.

Although the elimination rate of TCDD is 2- or 3-fold greater in the hamster than in the rat and mouse, this does not entirely explain the 10- to 100-fold difference in acute toxicity between the hamster and other rodent species. The guinea pig is most sensitive to the acute effects of TCDD, and it is the species with the slowest metabolism and elimination of TCDD, suggesting that toxicokinetics in part explains the unique sensitivity of the guinea pig to the acute toxicity of TCDD and TCDF (Van den Berg *et al.*, 1994).

## MECHANISM OF ACTION

The AhR is a ligand-activated transcription factor that is involved in the regulation of a number of genes, including those for enzymes that play a role in the metabolism of xenobiotics as well as genes involved in cell growth regulation and differentiation (Okey *et al.*, 1994; Safe, 1994; Hahn, 1998, 2002; Denison *et al.*, 2002; Denison and Nagy, 2003; Mandal, 2005). The AhR plays an important role in the altered gene expression and species- and tissue-specific toxicity resulting from exposure to specific PCB, PCDD, and PCDF congeners. It is well established that the majority of the toxic effects attributed to TCDD and TCDD-like chemicals require activation of the AhR. The toxicity of individual congeners is closely related to the affinity with which these compounds bind to the AhR, with the most toxic compounds being those that bind with the greatest affinity (Okey *et al.*, 1994). There are large species and strain differences in sensitivity to TCDD and related chemicals. Mouse and rat strain differences in sensitivity to TCDD can be partially explained by differences in the ligand binding affinity of their polymorphic AhR variants. A study in birds indicated that species differences in sensitivity to TCDD and related chemicals could be due, at least in part, to differences in amino acid composition of the ligand binding domain of the AhR (Karchner *et al.*, 2006). However, differences in AhR concentration or binding affinity do not fully explain the differences in species susceptibility to TCDD and TCDD-like chemicals (Denison *et al.*, 1986).

The AhR is a basic helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) containing transcription factor (Denison *et al.*, 2002; Denison and Nagy, 2003; Bradshaw and Bell, 2009). In the absence of a ligand, AhR occurs as a soluble multiprotein complex in the cytosol of the cell. The chaperone proteins are two molecules of hsp90 (a heat shock protein of 90 kDa), the X-associated protein 2 and p23 (a co-chaperone protein of 23 kDa). When TCDD or another ligand diffuses across the plasma membrane and binds to the AhR, the ligand AhR complex undergoes a

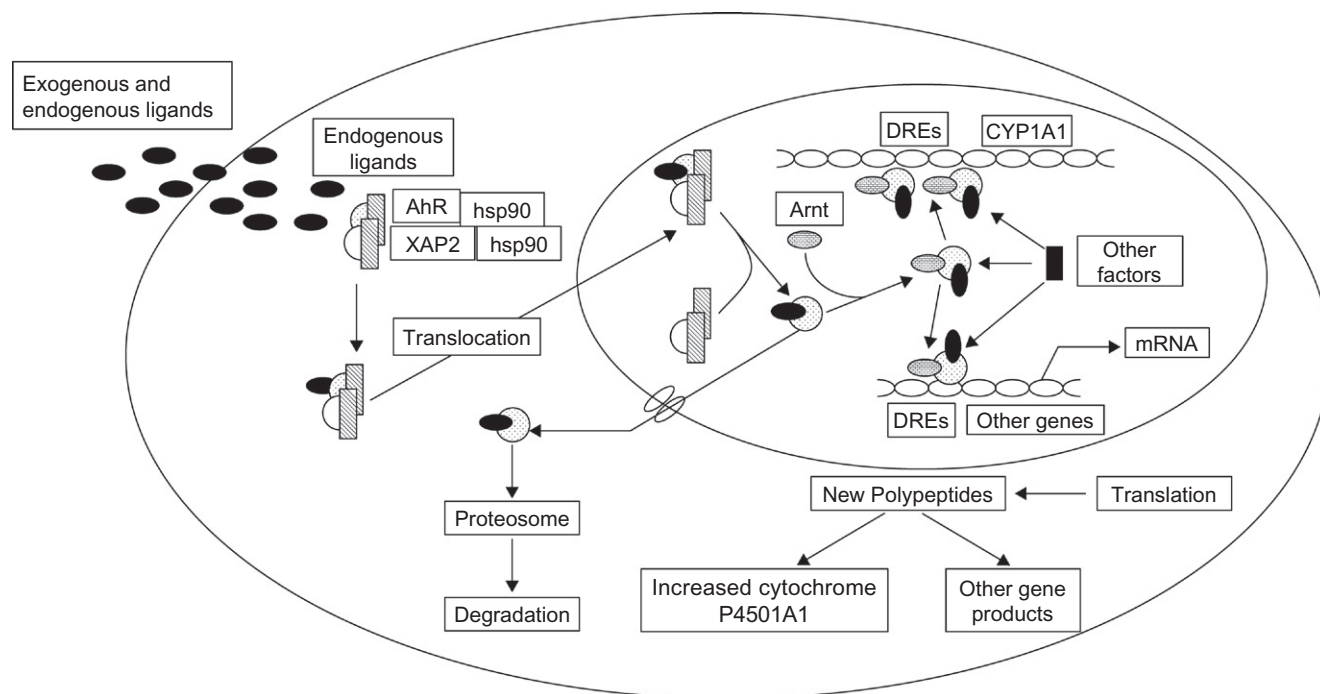


FIGURE 62.2 The proposed molecular mechanism of action of 2,3,7,8-TCDD and TCDD-like chemicals. See text for details.

conformational change that exposes a nuclear localization sequence (Figure 62.2). The complex translocates into the nucleus of the cell, and the chaperone proteins dissociate from the complex. The AhR–ligand then binds to the bHLH–PAS nuclear protein, AhR nuclear translocator, or Arnt. The formation of this heterodimer initiates conversion of the complex into a form that binds to DNA with high affinity on a specific recognition site called the dioxin responsive element (DRE). Binding of the ligand–AhR–Arnt complex to the DRE stimulates transcription of genes encoding cytochrome P450 enzymes in the CYP1A1 subfamily and other AhR-responsive genes that are located upstream of the DRE (Denison *et al.*, 2002; Denison and Nagy, 2003). Continuous and inappropriate modulation of gene expression is thought to be responsible for a series of biochemical, cellular, and tissue changes that result in toxicity characteristic of TCDD and related compounds (Denison and Heath-Pagliuso, 1998; Mandal, 2005).

## TOXICITY

### PBBs

None of the effects caused by PBBs are unique to this particular group of brominated congeners, but they are

characteristic of polyhalogenated hydrocarbons, including PBBs, PCBs, PCDDs, and PCDFs. The acute oral LD<sub>50</sub>s of FireMaster BP-6 as well as octabromobiphenyl and decabromobiphenyl are 2–22 g/kg body weight for a number of species. Death resulting from acute exposures can occur up to 2 weeks after dosing (DiCarlo *et al.*, 1978; Fries, 1985). In general, prolonged exposure to low concentrations of PBBs causes more deleterious effects than does exposure to a large, single dose (Damstra *et al.*, 1982). In most toxicity studies conducted with PBBs, the characteristic effects were a decrease in feed consumption with a concomitant decrease in body weight loss or body weight gain, which may not be entirely related to the decrease in feed consumption. Milk production and egg production may also decrease as a result, at least in part, of reduced feed consumption. The most prominent gross morphological and histological changes occurred in the liver of most species examined, with the exception of cattle, in which the kidney appeared to be the target organ (Damstra *et al.*, 1982; Fries, 1985). Hepatic lesions were characterized by swelling and vacuolation of hepatocytes with fatty infiltration (Corbett *et al.*, 1978; Kimbrough *et al.*, 1978; Gupta and Moore, 1979; Gupta *et al.*, 1981; Fries, 1985). Renal lesions in cattle included dilatation of collecting ducts and convoluted tubules as well as degeneration of tubular epithelium (Moorehead *et al.*, 1977; Fries, 1985). Both the thymus and the avian bursa of Fabricius decreased in size in

some species at relatively low doses. There is a loss of cells specific to the respective tissue and a disruption of the normal architecture (Gupta and Moore, 1979; Gupta *et al.*, 1981; Dharma *et al.*, 1982; Fries, 1985). In addition to reported atrophy of lymphoid tissues in a variety of species, there is evidence that exposure to PBBs causes suppression of the immune system (Moorehead *et al.*, 1977; Kimbrough *et al.*, 1978; Gupta and Moore, 1979; Gupta *et al.*, 1981, 1983; Fries, 1985). However, doses sufficient to cause other toxic effects are usually required to induce immunosuppression, and there appears to be differences between species in terms of sensitivity (Damstra *et al.*, 1982; Fries, 1985). A number of studies have demonstrated PBB-induced reproductive effects in a variety of species, including chickens (Ringer and Polin, 1977; Polin and Ringer, 1978b), Japanese quail (Babish *et al.*, 1975), rats (Corbett *et al.*, 1975; Beaudoin, 1977; Damstra *et al.*, 1982), mice (Corbett *et al.*, 1975; Preache *et al.*, 1976; DiCarlo *et al.*, 1978; Damstra *et al.*, 1982), rhesus monkeys (Allen and Lambrecht, 1978; Damstra *et al.*, 1982), and mink (Aulerich and Ringer, 1979). Commercial PBB mixtures have been shown to cause liver cancer in rats and mice (Kimbrough *et al.*, 1978, 1981; Damstra *et al.*, 1982; Gupta *et al.*, 1983; Fries, 1985).

### PCBs, PCDDs, and PCDFs

Exposure to PCBs, PCDDs, and PCDFs has been linked to a broad spectrum of effects, both *in vivo* and *in vitro*, that vary depending on method/age of exposure, sex of the individual, and dose/duration of exposure (Safe, 1994; Steinberg *et al.*, 2008). The majority of effects caused by commercial PCB mixtures are the same as those induced by TCDD because the responses caused by commercial PCB mixtures are due, in part, to the individual non-*ortho* coplanar and mono-*ortho* coplanar PCBs present in these mixtures that act as AhR agonists. Because some of the mono-*ortho* coplanar PCBs are present in relatively high concentrations in commercial mixtures and environmental extracts, this class of PCBs may contribute significantly to the TCDD-like activity of PCB mixtures (Safe, 1994).

A number of reports describe the common effects induced by TCDD and related PCDDs, PCDFs, and PCBs. The only major difference in these compounds is their relative toxic potencies. These effects are mediated through initial binding of the ligand to the AhR and are dependent on dose, age, sex, species, and strain of animal. In most cases, if a given species is more sensitive to a given class of compound (e.g., PCBs), this species will also be sensitive to the other classes (PCDDs and PCDFs). It has also been observed that in most instances, young animals are more sensitive than adults and females more sensitive than males. In

general, chickens, guinea pigs, and mink are the most sensitive species of animals to the toxic effects induced by 2,3,7,8-TCDD and TCDD-like chemicals. In contrast, hamsters and amphibians appear to be fairly resistant to the toxic effects. The effects include acute lethality, wasting syndrome, thymic and splenic atrophy, impairment of immune responses, hepatotoxicity and porphyria, chloracne and related dermal lesions, tissue-specific hypo- and hyperplastic responses, disruption of multiple endocrine pathways, carcinogenesis, teratogenicity, and reproductive toxicity (Poland and Knutson, 1982; McConnell, 1985; Safe, 1986, 1990, 1998; Dickson and Buzik, 1993; Fries, 1995; Giesy and Kannan, 1998; Schecter *et al.*, 2006).

TCDD causes lethality at very low doses in specific species. The oral LD<sub>50</sub> values for various species are as follows: 0.6–2.0 µg/kg body weight for the guinea pig, 22–45 µg/kg for the rat, 25–50 µg/kg for the chicken, 70 µg/kg for the monkey, 115 µg/kg for the rabbit, 100–200 µg/kg for the dog, 114–284 µg/kg for the mouse, and 1157–5000 µg/kg for the hamster (Safe, 1990). There is a delayed appearance of lethality, irrespective of dose. Death typically does not occur earlier than 1 week after exposure, and it may not occur until 6 weeks of exposure. Before death, the exposed animal generally undergoes a rapid and substantial weight loss called “wasting syndrome” (McConnell, 1985; Pohjanvirta and Tuomisto, 1994). This is accompanied by decreased food and water intake, which accounts for some, but not all, of the weight loss. These may be the only signs observed in rats, mice, guinea pigs, mink, and poultry after oral administration. However, skin lesions have been reported in hairless mice, and acne-like lesions have been described in the ears of rabbits after local application. Occasionally, poultry may show an increase in body weight prior to death due to accumulation of body fluids (subcutaneous edema, ascites, hydrothorax, and hydropericardium). In addition to body weight loss, the clinical syndrome in monkeys and cattle is characterized by skin and eyelid lesions and abnormal growth of finger- or toenails or hooves. The lesions in monkeys are follicular dermatitis (acne) of the face, neck, and forearms; enlarged tortuous Meibomian glands in the eyelid; and overgrowth and loss of nails of the hands and feet. Alopecia may be present, particularly in the areas of the body showing dermatitis. The skin of cattle is thickened and dry, particularly over the neck, shoulders, and back (McConnell, 1985).

TCDD is a highly immunosuppressive chemical in laboratory animals. Effects include decreased host resistance to infectious disease and suppressed humoral and cell-mediated immune responses. Both pre- and post-natal exposure of mice and guinea pigs reduced both the delayed hypersensitivity and lymphoproliferative response. TCDD appears to selectively interfere with



antigen-specific activation of T cells. In addition to immune suppression, TCDD promotes inflammatory responses (Dickson and Buzik, 1993; Pohjanvirta and Tuomisto, 1994; Mandal, 2005).

Thymic atrophy is one of the most uniform and consistent findings in TCDD-exposed mammals. It consists of depletion of small immature cortical thymocytes, making a distinction between cortex and medulla difficult. Although both pyknotic and scattered necrotic lymphocytes have been reported, frank necrosis is not a typical feature of the lesion. At less toxic doses, the thymus might look normal histopathologically while being one-half normal size (McConnell, 1985; Pohjanvirta and Tuomisto, 1994).

TCDD exposure results in hepatomegaly in all species investigated, but there is extensive variation among species in the severity of this lesion. The changes in the liver are accompanied by altered liver function characterized by enzyme induction, porphyria, impaired plasma membrane function, and hyperlipidemia. Liver lesions are most severe in the rabbit, which displays extensive necrosis. In other species, such as the rat, mouse, mink, and, to a lesser extent, the guinea pig, hamster, bovine, and monkey, the predominant features are hepatocellular hypertrophy, multinuclear hepatocytes, steatosis, and inflammatory cell infiltration, often accompanied by scattered focal necrosis with a preferentially centrilobular location. Female rodents are more susceptible to the hepatic effects of TCDD compared to males (McConnell, 1985; Dickson and Buzik, 1993; Pohjanvirta and Tuomisto, 1994; Safe, 1994).

Intrahepatic bile duct hyperplasia has been described in rodents and monkeys, but it is a more prominent feature in chronically exposed animals. Marked epithelial proliferation of the extrahepatic bile duct and gallbladder has been described in monkeys and cattle. Epithelial erosions, ulcers, and inflammation are characteristic components of the lesion. Hyperplasia of the epithelium lining of the urinary tract has been described in guinea pigs, cattle, and monkeys. The lesion extends from the renal pelvis to the urinary bladder, stopping at the level of the urethra. Monkeys exposed to TCDD-like compounds develop a lesion referred to as "simian gastropathy." In acute lethal exposures, the chief (acid-producing) cells are replaced by hyperplastic mucous-producing cells. In more chronic exposures, the hyperplastic change becomes more pronounced and at times appears to invade adjacent tissues. A similar but much less severe lesion has been described in rats exposed to PBBs. The large intestine also shows hyperplastic changes in monkeys chronically exposed to these chemicals (McConnell, 1985).

The skin and associated structures in monkeys, rabbits, and certain strains of mice show characteristic lesions as a result of exposure to TCDD and related

chemicals. In monkeys, the lesion is characterized microscopically by mild epithelial hyperkeratosis and severe atrophy of sebaceous glands and hyperkeratosis of their ducts. The ducts become occluded with keratinaceous debris and grossly the lesion mimics an acne-like lesion. A similar lesion is observed in the Meibomian glands of the eyelid and ceruminous glands of the external auditory canal. Alopecia, dry scaly skin, and fingernail loss are also features of exposure in monkeys. Although most strains of mice do not show skin lesions, certain "hairless" strains show a similar skin lesion as that in monkeys. The inner surface of a rabbit's ear also develops acne-like lesions if the compound is applied directly to the skin. Cattle also show a characteristic skin disease when exposed to these compounds (McConnell, 1985; Safe, 1994).

TCDD and related chemicals reduce serum thyroxine ( $T_4$ ) concentrations rapidly in rats. Changes in  $T_4$  concentrations in other species are less consistent. The decrease in serum  $T_4$  concentrations is thought to be due to accelerated clearance of the hormone. In addition to altering thyroid hormone concentrations, TCDD appears to modulate the concentration of thyroid hormone receptors. Other endocrine changes include alterations in corticosteroid concentrations and in the concentrations of hormones associated with the reproductive system in rodents, monkeys, and birds (Pohjanvirta and Tuomisto, 1994; Safe, 1994; Brouwer *et al.*, 1998; Ulbrich and Stahlmann, 2004).

It is assumed that the interaction of TCDD with the AhR regulates a number of genes, although CYP1A1 has been studied most thoroughly. Thus, in addition to monooxygenases dependent on cytochrome P4501A1, a number of other enzymes are induced (Pohjanvirta and Tuomisto, 1994; Huwe, 2002).

Effects on the nervous system are manifested as hyperactivity, impaired learning, changes in gender-related behavior, and changes in brain neurochemical concentrations in rats, mice, and monkeys (Safe, 1994; Chen and Hsu, 1994; Schantz *et al.*, 1995; Rice, 1999; Berger *et al.*, 2001; Wang *et al.*, 2002; Salama *et al.*, 2003; Ulbrich and Stahlmann, 2004; Branchi *et al.*, 2005).

TCDD and approximate stereoisomers have been shown to affect female reproductive endpoints in a variety of animal studies. Among the effects reported are a decrease in the number of females mated in rats, mink, and monkeys; fewer completed pregnancies in rats, mink, and monkeys; lower maternal weight gain during pregnancy in rats, rabbits, and monkeys; decreased litter size in rats, rabbits, mink, and swine; effects on external genitalia and gonads in rats, mice, and guinea pigs; altered estrous and menstrual cycles in rats, mice, and monkeys; and delayed maturation of the mammary gland and increased incidence of breast cancer in rats and mice. Decreased egg production and hatchability occur in a number of avian species. Numerous

studies have indicated that TCDD and related chemicals are anti-estrogenic presumably due to increased metabolism of estrogen and a decreased number of estrogen receptors (Golub *et al.*, 1991; Ronnback, 1991; Peterson *et al.*, 1993; Safe, 1994; Brown and Lamartiniere, 1995; Gray and Otsby, 1995; Gray *et al.*, 1997; Brown *et al.*, 1998; Fenton *et al.*, 2002; Ishimura *et al.*, 2002a,b; Muto *et al.*, 2002; Salisbury and Marcinkiewicz, 2002; Vorderstrasse *et al.*, 2004; Fenton, 2006; Jablonska *et al.*, 2010). In males, TCDD and related compounds decrease testis and accessory sex organ weight, cause abnormal testicular morphology, decrease spermatogenesis, and reduce fertility when given to adult animals in doses sufficient to reduce feed intake and/or body weight. Some of these effects have been reported in chickens, rhesus monkeys, goats, guinea pigs, rats, and mice treated with overtly toxic doses of TCDD or TCDD-like chemicals. Effects of TCDD on the male reproductive system are thought to be due in part to an androgen deficiency. The deficiency in rats is caused by decreased plasma testosterone and dihydrotestosterone concentrations and unchanged plasma clearance of androgens and luteinizing hormone (LH) (Dickson and Buzik, 1993; Peterson *et al.*, 1993; Safe, 1994; Fielden *et al.*, 2001; Hsu *et al.*, 2003, 2007; Kuriyama and Chahoud, 2004; Oskam *et al.*, 2005; Foster *et al.*, 2010).

In most laboratory mammals, gestational exposure to TCDD produces a characteristic pattern of fetotoxic responses that consist of thymic hypoplasia, subcutaneous edema, decreased fetal growth, and prenatal mortality. In addition to these common fetotoxic effects, there are other effects of TCDD that are highly species specific, including cleft palate formation in the mouse and intestinal hemorrhage in the rat. In the mouse, hydronephrosis is the sensitive sign of prenatal toxicity, followed by cleft palate formation and atrophy of the thymus at higher doses and by subcutaneous edema and mortality at maternally toxic doses. In the rat, TCDD prenatal toxicity is characterized by intestinal hemorrhage, subcutaneous edema, decreased fetal growth, and mortality. Structural abnormalities occur in the rat only at relatively large doses. In the hamster fetus, hydronephrosis and renal congestion are the most sensitive effects, followed by subcutaneous edema and mortality. In the rabbit, an increased incidence of extra ribs and prenatal mortality is found, whereas in the guinea pig and rhesus monkey, prenatal mortality is seen (Birnbaum *et al.*, 1989; Couture *et al.*, 1990; Dickson and Buzik, 1993; Peterson *et al.*, 1993).

Based on LD<sub>50</sub> values, avian embryos are more sensitive to toxicity of TCDD and related compounds compared to mammals. Among bird species, most of the developmental toxicity research has been done on the chicken, which is considered to be the most sensitive avian species to TCDD-like chemicals. Injection of

TCDD or its approximate stereoisomers into fertilized chicken eggs causes a toxicity syndrome in the embryo characterized by pericardial and subcutaneous edema, liver lesions, inhibition of lymphoid development in the thymus and bursa of Fabricius, microphthalmia, beak deformities, cardiovascular malformations (cardiac dilation, thinner ventricle walls, and reduced responsiveness to chronotropic stimuli), and mortality. Clinical signs in turkey embryos include microphthalmia, beak deformities, and embryo mortality but not liver lesions, edema, or thymic hypoplasia, whereas ring-necked pheasant embryos experience only mortality. Thus, the clinical signs of toxicity of TCDD and its approximate stereoisomers are species dependent, with embryo mortality being the only common effect (Peterson *et al.*, 1993; Powell *et al.*, 1996; Kopf and Walker, 2009; Cohen-Barnhouse *et al.*, 2011).

Normal development of male reproductive organs and imprinting of typical adult sexual behavior patterns require that sufficient testosterone be secreted by the fetal and neonatal testes at critical times in early development before and soon after birth. Perinatal exposure of male rats to TCDD can produce both prenatal and postnatal androgenic deficiencies that are manifested as a decrease in anogenital distance; a delay in testis descent; decreased plasma concentrations of testosterone, 5 $\alpha$ -dihydrotestosterone, and LH; and a decrease in the weights of the seminal vesicles and the ventral prostate. Perinatal exposure of the rat to TCDD also results in decreased spermatogenesis as well as decreased testis and epididymis weights. In addition, demasculinization and feminization of sexual behavior has been reported (Golub *et al.*, 1991; Peterson *et al.*, 1993).

There have been several long-term bioassays for carcinogenicity of TCDD in several species, with all studies producing positive results. Studies of Sprague-Dawley rats indicated an increased incidence of hepatocellular hyperplastic nodules and hepatocellular carcinomas in female rats but not in male rats. Additional lesions noted were squamous cell carcinoma of the tongue and nasal turbinates/hard palate in both sexes. In addition to the liver, tongue, nasal turbinates, and hard palate, increased incidences of lung tumors were observed in females (Kociba *et al.*, 1978; Lucier *et al.*, 1993). In a study of Osborne-Mendel rats and B6C3F1 mice, TCDD induced malignant liver tumors in female rats and male and female mice. The incidence of thyroid gland tumors increased in male rats, whereas TCDD-induced neoplasms of the adrenal gland occurred in both male and female rats. Fibrosarcomas of the subcutaneous tissue occurred in both female rats and mice. Lymphomas and lung tumors were also observed in female mice (NTP, 1982; Lucier *et al.*, 1993). In the male Syrian golden hamster, exposure to TCDD resulted in squamous cell carcinomas of the skin in the facial region,

with some of the lesion metastasizing to the lung (Rao *et al.*, 1988; Lucier *et al.*, 1993). B6C3 and B6C mice of both sexes administered TCDD developed thymic lymphomas and hepatic neoplasms (Della Porta *et al.*, 1987; Lucier *et al.*, 1993).

The studies cited previously that are summarized by Lucier *et al.* (1993) indicate that TCDD is a multisite carcinogen in both sexes, and in several species it is a carcinogen in sites remote from the site of treatment and it increases cancer incidence at doses below the maximum tolerated dose. In two-stage models for liver and skin cancer, it is clear that TCDD is a potent promoting agent with weak or no initiating activity. The consensus is that TCDD is an example of a receptor-mediated carcinogenesis in that (1) interaction with the AhR appears to be a necessary early step; (2) TCDD modifies a number of receptor and hormone systems involved in cell growth and differentiation, such as epidermal growth factor receptor and the estrogen receptor; and (3) hormones exert a profound influence on the carcinogenic actions of TCDD. Although tumor promotion data are limited for PCDFs and coplanar PCBs, it appears that these chemicals are liver tumor promoters with potencies dependent on their binding affinity to the AhR (Lucier *et al.*, 1993; Schwarz and Appel, 2005).

## TREATMENT

The PBB incident in Michigan that resulted in the contamination and subsequent disposal of thousands of animals and millions of pounds of contaminated food products because of the long biological half-lives of these compounds prompted investigation of ways to enhance elimination of persistent PHAHs. Strategies reported in Fries (1985) included activated charcoal in rats and cows (Cook *et al.*, 1978; McConnell *et al.*, 1980); mineral oil in rats and monkeys (Kimbrough *et al.*, 1980; Rozman *et al.*, 1982); high fiber diets in rats (Kimbrough *et al.*, 1980); phenobarbital in cows (Cook *et al.*, 1978); and colestipol, mineral oil, propylene glycol, or petroleum jelly with or without restricted feeding in chickens (Polin *et al.*, 1985, 1989). In general, none of these elimination enhancement strategies proved to be effective despite the fact that they were employed for periods of 3–6 months (Fries, 1985). Other studies have examined different strategies to increase clearance of dioxins from animals. Rats fed clenbuterol-supplemented feed for 10 days after exposure to TCDD had 30% less fat than control rats and a 30% decrease in the body burden of TCDD. In other studies, rats and mice fed dietary fiber, chlorophyll, or an insoluble evacuation substance (chlorophyllin-chitosan)

had significantly increased excretion of PCDDs and PCDFs and reduced TEQ body burden (Huwe, 2002).

As suggested previously, once animals have been contaminated with the persistent PHAHs, there are currently no practical methods available to quickly reduce body burdens. Therefore, in a contamination incident, products are removed from the market and animals may have to be destroyed. A common strategy to reduce concentrations of contaminants in exposed animals is to provide the animals with uncontaminated feed and withhold products from the market until concentrations of the contaminant have decreased to an acceptable level. In the case of the persistent PCBs, PBBs, and PCDDs, long half-lives in animals require long withdrawal periods. Estimates of the half-lives of PCBs/PCDDs/PCDFs in milk range from 40 to 190 days. In beef cattle adipose tissue, half-lives are in the range of 100 to 200 days. The half-lives of PCDDs in adipose tissue and eggs of chickens range from 25 to 60 days. Depuration is the only way to reduce body burdens, but it may be uneconomical in many situations because of the length of time required (Huwe, 2002).

Currently, the best way to reduce PCB/PCDD/PCDF concentrations in livestock is to minimize exposure. In general, the substitution of plant meals for animal and fish meals may prove to be an effective strategy to lower contaminant intake in livestock and aquaculture (Huwe, 2002).

## CONCLUSIONS AND FUTURE DIRECTIONS

The PHAHs, which include PCBs, PBBs, PCDDs, and PCDFs, are environmentally persistent, lipophilic compounds that have a tendency to bioaccumulate and biomagnify at the higher levels of the food chain. Concentrations of these chemicals have been detected in remote areas of the world and in a variety of animal species, including humans. Although certain of these chemicals can pose a very serious threat to the health of animals and humans, exposure situations are generally such that risks of health effects are low. The most significant problem for those involved in producing a safe food supply is contamination of food products beginning at the animal. In addition, there are areas of the environment that are heavily contaminated by these chemicals because of past industrial activities. Animals and humans residing in or near contaminated locations are certainly at risk for serious health effects. In these situations, efforts must continue to eliminate or reduce exposure to these very persistent and toxic chemicals.



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## Polycyclic aromatic hydrocarbons

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### INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) constitute a large family of organic compounds with two or more fused aromatic rings. They are semivolatile, sparingly soluble in water, highly lipophilic, and high-molecular-weight compounds. PAHs are adsorbed on particles in the air, soil, water, and sediment. These compounds are not released into the environment as single compounds; instead, they often occur as mixtures. Some representative PAHs that are present in the environment are shown in Figure 63.1.

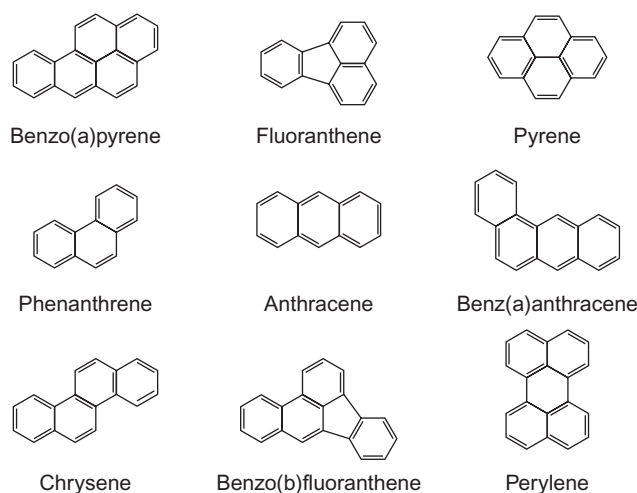


FIGURE 63.1 Representative PAH compounds encountered in the farm environment.

PAHs are formed mainly as a result of pyrolytic processes, especially the incomplete combustion of organic materials during industrial and other human-initiated activities, such as processing of coal and crude oil and coal-fired power generation. Other sources of PAH contamination of the environment and exposure to humans, wildlife, and farm animals include industrial and automobile emissions, hazardous waste sites, refuse burning, wildfires, arson, and warfare (Ramesh *et al.*, 2004; Hood *et al.*, 2009). Both food ingestion and inhalation represent the major routes of PAH exposure to humans and livestock. From a food safety perspective, the contamination of food products of animal origin (milk, butter, cheese, meat, etc.) by PAHs has received considerable attention because these products provide nourishment to humans. Humans are exposed to PAHs either directly or indirectly. The direct pathways for human exposures to PAHs include ingestion of soil (accidentally by children) or vegetables. Secondary or indirect pathways include those through which humans are exposed to PAHs via the consumption of products of livestock, which themselves ingest PAHs by grazing on contaminated pastures and accidental consumption of soil (Hartemann, 2004).

The increasing contamination of the farm environment by these toxicants has raised awareness concerning possible risks to livestock and ultimately to the consumer. However, compared to their aquatic counterparts, the toxicity of PAHs to farm animals has not been studied in detail. Given the severity of global environmental contamination by PAHs in diverse matrices such as air, water, soil, roughage, and sediment (Ramesh *et al.*, 2011) and their environmental persistence (a half-life of



TABLE 63.1 Concentrations of PAHs in grass samples

Plant type	Concentration (mg/kg dry grass)	Sampling site	References
Plantain	200–1700	Urban area (NL)	Bakker <i>et al.</i> (2000)
Grass	1461	Highway (France)	Bryeselbout <i>et al.</i> (2000)
Grass	900	Highway (France)	Crépineau-Ducoulombier <i>et al.</i> (2003)
Grass	25	Remote pasture	Crépineau-Ducoulombier <i>et al.</i> (2003)
Ryegrass	<1	Highway (France)	Crépineau-Ducoulombier <i>et al.</i> (2004), Dan-Badjo <i>et al.</i> (2007, 2008)
Grass	153	Urban area (UK)	Meharg <i>et al.</i> (1998)
Grass	142	Road side	Muller <i>et al.</i> (2004)
Ray grass	100–900	Rural area (UK)	Smith <i>et al.</i> (2001)
Fesche	136–510	Rural area (UK)	Smith <i>et al.</i> (2001)

TABLE 63.2 Concentrations of PAHs in agricultural soil samples<sup>a</sup>

Location	Concentration (mg/kg soil dry wt)	Reference
Hisar, India	30–78	Bishnoi <i>et al.</i> (2009)
El Paso, Texas, USA	0.0–0.63	De La Torre-Roche <i>et al.</i> (2009)
Ebro river basin, Spain	0.001–0.47	Hildebrandt <i>et al.</i> (2007)
Poland	0.30–0.60	Maliszewska-Kordybach <i>et al.</i> (2009)
Valasske Mezinci, Czech Republic	0.86–10,840	Plachá <i>et al.</i> (2009)
Taizhou, China	0.26–3.4	Tang <i>et al.</i> (2010)

<sup>a</sup>Agricultural soil refers to soil that is used for agricultural operations, such as soil for food production (raising crops), pasture land, and grassland.

100 days in soil; Jurjanz *et al.*, 2008), higher mammals, including farm animals, are at an increased risk of PAH exposure, accumulation, and the adverse health effects that may ensue. Unfortunately, to date, no efforts have been made to provide a compendium of information on PAH intake, behavior, and biological fate in farm animals. This chapter is an attempt to summarize the key research findings in this area, the data gaps, and scope for future studies.

## EXPOSURE OF FARM ANIMALS TO PAHS

As a consequence of rapid industrialization, PAHs are released into the environment on a large scale (Ramesh *et al.*, 2011). The contamination of pastures by PAHs occurs via atmospheric deposit (dry or wet deposition of particles onto leaf surfaces; Wild *et al.*, 1992) and root absorption (Su and Zhu, 2008) of these compounds. Pastures close to highways (vehicular traffic) and industries register greater concentrations of PAHs (Dan-Badjo *et al.*, 2007). In this context, high levels of PAHs occur in grass, with levels ranging from 25 mg/kg to 1 g/kg dry weight (wt) (Table 63.1). Cattle that graze on pastures near highways and airports have been reported to ingest

2 mg of PAHs/animal/day (Crépineau-Ducoulombier *et al.*, 2004).

Soil is another important route of exposure to PAHs during grazing. The concentrations of PAHs in exposed soils are estimated to be more than 70 mg or 10,000 mg/kg dry wt (Table 63.2). Soil that is rich in clay-humus material (high organic carbon content) sequesters PAHs, which are not amenable to hydrolysis and microbial degradation (Verstraete and Devliegher, 1996). If the average intake of soil is approximately 0.5 kg/animal/day (Fries, 1996), soil ingestion contributes approximately 50 mg of PAHs into each animal/day (Bryeselbout *et al.*, 2000). Therefore, soil, in addition to grass, is another matrix that contributes to substantial PAH intake.

In addition to pastures and soil, the farm environment as a whole has been reported to be contaminated with PAHs. Common sources of farm animal exposure to PAHs are feedstuffs, drinking water, barn dust, and manure. The concentrations of PAHs reported were 210 µg/kg, 2150 µg/kg, 543 µg/kg, and 52 ng/L for feedstuffs, dust, manure, and drinking water, respectively (Raszyk *et al.*, 1998). The outdoor and indoor air for livestock dwellings was contaminated by PAHs; the concentrations for pig and cow houses were 3 and 9 ng/m<sup>3</sup> and 4 and 6 ng/m<sup>3</sup>, respectively (Cigánek *et al.*, 2002; Cupr *et al.*, 2005). Based on PAH concentrations in farm, barn, and cattle houses, Cigánek *et al.* (2002) calculated that

**TABLE 63.3** Total PAH exposure in farm animals: comparison between pigs and cows

	Pigs	Cows
Feeds ( $\mu\text{g}/\text{day}$ )	256	14,156
Water ( $\mu\text{g}/\text{day}$ )	0.80	1.5
Inspired air ( $\mu\text{g}/\text{day}$ )	0.25	2.2
Sum of intake ( $\mu\text{g}/\text{day}$ )	257	14,160
Exposure ( $\mu\text{g}/\text{kg}/\text{day}$ )	2.6	35

Reproduced from Cigánek *et al.* (2002) with permission of the Czech Academy of Agricultural Sciences.

PAH exposure is 20 times higher in cows than in pigs (Table 63.3).

Livestock may also be exposed inadvertently to accidental release of PAHs near oil and natural gas exploration and production sites that emanate these contaminants along with petroleum hydrocarbons (Pattanayek and DeShields, 2004). Accidental spillage of coal ash from coal-fired electric power plants and the atmospheric transport of coal ash plumes (Hood *et al.*, 2011) to farms in the catchment areas constitute another source of exposure for farm animals to PAHs through inhalation and ingestion. The absorption of PAHs through dermal contact in livestock is considered negligible compared to ingestion of fodder and soil (Jurjanz *et al.*, 2008).

### UPTAKE, DISPOSITION, BIOACCESSIBILITY, AND BIOAVAILABILITY OF PAHS IN FARM ANIMALS

As mentioned previously, feed is the major source of PAHs for livestock – that is, up to 99% (Cigánek *et al.*, 2002). The dietary habits determine the levels of PAH intake. Domesticated dairy and meat animals are usually raised in confinement (indoors) or on pastures. Whereas animals such as pigs and rabbits are housed indoor, cows, goats, sheep, and horses graze on farmlands (that could be polluted) with occasional supplementation with feed concentrates. Bioaccessibility and bioavailability studies are conducted by various research groups to demonstrate the transfer of PAHs from feed matrices to the digestive tract, milk, and eventually the general circulation. When PAH intake from complex matrices such as soil by rats and farm animals is involved, the terms bioaccessibility and bioavailability have been used interchangeably in the literature, causing confusion to readers. For the sake of clarity, both terms are defined here. Whereas bioaccessibility refers to the fraction of toxicant

intake that is available for absorption in the gastrointestinal tract, bioavailability refers to the fraction of administered dose of a toxicant that reaches the systemic circulation (blood compartment) from the gastrointestinal tract (Paustenbach, 2000).

### Transfer of PAHs in cows

As part of larger studies that are directed at unraveling the mechanisms that govern the chemical behavior and fate of PAHs, Costera *et al.* (2010) conducted studies to access the release of PAHs from grass in the digestive tract of ruminants. Three cows fitted with a rumen fistula were used to introduce six nylon bags containing  $4\mu\text{g}$  PAHs/g dry wt of grass containing phenanthrene (Phe), pyrene (Pyr), and benzo(*a*) pyrene (BaP) into the rumen of each animal. The bags were incubated for time periods of 1, 3, 6, 12, 24, and 48 h. The disappearance rates for the PAHs administered were greater than 90, 81, and 80% for Phe, Pyr, and BaP, respectively, whereas for grass it was 20–60%. These studies revealed a rapid disappearance rate for PAHs in ruminants, whereas the feed matrix (grass, which served as a carrier for PAHs) had no effect on the bioavailability of PAHs.

Among farm animals, cows ingest a greater amount of PAHs (approximately  $2\text{mg}/\text{day}$ ) via roughage (Crèpineau-Ducoulombier *et al.*, 2004) by grazing on pastures close to highways and airports, where the PAH residue levels in soil were reported to exceed those in grass (Crèpineau-Ducoulombier and Rychen, 2003; Dan-Badjo *et al.*, 2007). Residual concentrations of PAHs up to  $100\text{--}200\mu\text{g}$  PAH/g of soil have been reported (Ramesh *et al.*, 2011), and if these values are extrapolated to consumption of soil by cattle, PAH intake of  $50\text{mg}/\text{day}$  can be expected. The mobilization of PAHs in soil during digestion (bioaccessibility) may play an important role in the disposition of PAHs in farm animals. To study PAH bioaccessibility in ruminants, Jurjanz and Rychen (2008) supplemented pasture soil with 5.4, 6.8, and  $8.4\text{mg}/\text{kg}$  of Phe, Pyr, and BaP, respectively. These PAHs were exposed to conditions that simulated the digestive compartments of the rumen, abomasums, and intestines. For all three PAH compounds studied, the bioaccessibility was more relevant for the rumen compartment than for the abomasum and intestine. Overall, Phe bioaccessibility was found to be higher compared to that of Pyr or BaP.

The aforementioned study demonstrated that soil-bound PAH parent compounds are bioaccessible in the ruminant digestive tract. This observation calls into question the possible transfer of these bioaccessible PAHs from the gastrointestinal tract to milk; if such a transfer occurs, would it be in the form of parent compound or metabolites? To investigate this question,

Lutz *et al.* (2006) administered soil fortified with fluorene (Flu; 104 µg/g dry wt of soil), Phe (82 µg/g), Pyr (78 µg/g), and BaP (33 µg/g) to dairy cows via rumen fistulae for 28 days. Whereas Phe, Flu, and Pyr have been found to be extensively metabolized and transferred to milk (as recorded by their metabolite concentrations in milk), BaP metabolites were not found to be secreted into milk. Mammary epithelium plays an important role in the transfer of PAH from food to milk. Using a bovine mammary cell line, Cavret *et al.* (2005a) demonstrated transepithelial permeability of <sup>14</sup>C-labeled BaP, Pyr, and Phe. These studies revealed that Phe and Pyr were able to cross mammary cell layers, whereas BaP was not. The differences in transfer rates support the contention that the lipophilicity and molecular weight of PAH govern their transport across the mammary epithelium. These findings demonstrate that subsequent to biotransformation, the unchanged parent compound (if any) and metabolites are likely to be distributed to target tissues such as liver, intestine, adipose, and muscle and reproductive tissues, as shown for cattle (Cigánek and Neca, 2006) and sheep (Rhind *et al.*, 2011), or excreted through urine, as demonstrated for goats (Grova *et al.*, 2002).

Additional experiments by Cavret *et al.* (2003) employed Caco-2 (human colon carcinoma) cells to measure the transepithelial intestinal permeability for Phe and BaP. After a 6-h exposure, the absorption levels of Phe and BaP were 9.5 and 5.2%, respectively. Cavret *et al.* (2005b) also studied the uptake and transport of <sup>14</sup>C-labeled BaP, Pyr, and Phe in Caco-2 cells. Although all three PAHs were able to cross intestinal layers, Pyr and Phe, which have a lower lipophilicity, were found to rapidly cross the intestinal barrier compared to BaP, which is relatively more lipophilic. As a result, extensive metabolism of BaP occurs in the intestine, and accumulation of metabolites takes place in target tissues and they become bioavailable. In other words, the faster a PAH crosses the intestinal barrier, the less metabolized it will be in the intestine (Cavret *et al.*, 2004). Cavret and Feidt (2005) conducted studies on the transepithelial passage of BaP, Pyr, and Phe in Caco-2 cells. The intestinal transfers of BaP, Pyr, and Phe, respectively, were found to be 26, 4, and 2 times lower with CYP (1A1, 1B1, and 3A4) inhibitors. These studies provide additional evidence that intestinal metabolism of PAHs play a pivotal role in their bioavailability in higher mammals.

### Transfer of PAHs in pigs

To study whether PAH absorption in intestine occurs in concert with fat absorption, Laurent *et al.* (2001) administered pigs with [<sup>14</sup>C] Phe and [<sup>14</sup>C] BaP through milk and assayed their concentrations in blood. Both these compounds reached peak levels in blood within 5 or 6 h

after ingestion. The difference in molecular weights (Phe 178 vs. BaP 252) and water solubilities (Phe 1.2 mg/L vs. BaP 3.8 µg/L) notwithstanding, the absorption rates for these two compounds were found to be similar to that observed for fat absorption (Dubois *et al.*, 1996). Based on these findings, it appears that (1) transport of PAHs from milk to blood occurs through lipid phase rather than aqueous phase, and (2) the more lipophilic members of PAHs are poorly absorbed by the intestine from feed materials (Hoogenboom, 2005).

### Transfer of PAHs in sheep and goats

The study conducted by West and Horton (1976) was the first to demonstrate that lactational transfer of PAHs occurs in sheep. In this study, radiolabeled 3-methylcholanthrene and BaP were orally administered to sheep. No parent compound was present in milk, but the hydroxylated products were present. These compounds were metabolized from 33 to 55% (of the parent compound) within 24 h after administration. For both these chemicals, the proportion of water-soluble material (presumably metabolites) increased with time.

Grova *et al.* (2002, 2005) studied the excretion of PAHs in milk, urine, and feces of goats subsequent to a single oral administration of 5 µg/kg <sup>14</sup>C-Phe, <sup>14</sup>C-Pyr, and <sup>14</sup>C-BaP. The lactational transfer factors (percentage parent dose) were found to be 1.5, 1.9, and 2%, respectively, for the previously mentioned compounds. The urinary excretion rates for Phe, Pyr, and BaP were 40, 11, and 6%, respectively, whereas the fecal excretion rates for these compounds were 22, 20, and 88%, respectively. Perhaps the configuration of BaP (five benzene rings) compared to those of Phe (three benzene rings) and Pyr (four benzene rings) may have been responsible for the differential processing of these chemicals by goats. In a follow-up study conducted by this research group (Grova *et al.*, 2006), 20 µg PAHs/kg body wt was administered to goats for 28 days. Parent compounds and their hydroxylated metabolites were detected for most of the PAHs in milk except for BaP. Lapole *et al.* (2007) also documented similar behavior and fate for hydroxylated metabolites of these chemicals.

Similar to the studies conducted in cows, Costera *et al.* (2009) studied the bioavailability of Phe, Pyr, and BaP administered to goats through oil (castor oil polyether), hay, or soil. Because the weights of the animals were not provided, it is difficult to compute the exact doses of the chemicals administered. However, the amount of PAHs ingested by the goats varied from 17 to 50 mg. The authors found an increased excretion of PAHs through urine compared to milk. Among the three matrices used, the absorption rates (as reflected by the urine and milk excretion rates) were higher for hydroxylated

metabolites of Phe and Pyr when oil was used as a support matrix compared to hay and soil. Interestingly, the differences between soil and hay in the transfer kinetics of PAHs were negligible.

In a subsequent bioavailability study in goats, Ounnas *et al.* (2009) used three doses of the previously mentioned PAHs using soil and sunflower oil as matrices. The relative bioavailability of Phe and Pyr was found to be enhanced when oil was used as a vehicle instead of soil. Soil was reported to sequester PAHs (Bogan and Sullivan, 2003), thereby limiting the PAH fraction available for absorption. On the basis of PAH excretion rates through urine and milk, Ounnas *et al.* (2009) computed absorption rates of more than 30% for PAH parent compounds and metabolites in ruminants. These findings therefore indicate the need for factoring in soil ingestion parameter when assessing the PAH exposure risk to grazing ruminants, recognizing that cattle consume approximately 0.5 kg/animal/day (Fries, 1996).

## BIOTRANSFORMATION OF PAHS IN FARM ANIMALS

Absorbed PAHs into farm animals undergo a series of enzymatic reactions referred to as "biotransformation." Consequently, the toxicants become less lipophilic and more hydrophilic (polar) in the body to be excreted eventually. Drug-metabolizing enzymes possess different substrate specificities, and their expression levels are affected by exposure to PAHs as well as by physiological factors (Graham and Lake, 2008). Although biotransformation serves as a detoxification strategy, biotransformation enzymes generate stable metabolic products that are highly reactive and severalfold more toxic than the parent compounds. Some of the reactive intermediates (metabolites with a short half-life) exert local toxicity in the target tissues where they are generated. On the other hand, other intermediates (metabolites with a long half-life) may be produced in liver, released into the general circulation, and become bioavailable to affect distant tissues (Connelly and Bridges, 1980).

Although most PAHs have been suspected of causing toxicity, and a few of them of causing cancer, more than 100 types of PAHs are known to exist in nature. This necessitated using a surrogate PAH compound to gain comprehensive information on the mechanisms involved in the development of toxicity/cancer by these chemicals. BaP is well suited for this role because this chemical is considered a prototypical PAH compound, being the most extensively characterized and studied in terms of its toxicity. Here, we provide a snapshot

of metabolic biotransformation of BaP, which is relevant in understanding the causal factors involved in PAH toxicity.

Initial oxidation of BaP is catalyzed by the CYP450 family of enzymes (CYP1A1, CYP1A2, and CYP1B1), yielding arene oxides (9-OH-BaP, 7-OH-BaP, 6-OH-BaP, 3-OH-BaP, and 1-OH-BaP). These arene oxides rearrange to phenols or undergo hydration that is catalyzed by epoxide hydrolase (EH) with the resultant generation of BaP-9,10-diol, BaP-7,8-diol, and BaP-4,5-diol (Shimada and Guengerich, 2006). The previously mentioned metabolites (epoxides, hydroxy metabolites, and dihydrodiols) represent phase I metabolites of BaP. Phase I metabolism introduces more polar chemical groups such as hydroxy metabolites into the molecule. As a result, the molecule becomes more electrophilic, leading to increased reactivity. A good example of electrophilic metabolite formation is BaP 7,8-oxide, which is inactivated by microsomal epoxide hydrolase to 7,8-dihydrodiol. The 7,8-dihydrodiol serves as a substrate for a second monooxygenation step, which introduces a further epoxide moiety leading to the formation of a dihydrodiol bay region epoxide, termed the BaP 7,8-diol 9,10-epoxide (BPDE; Oesch *et al.*, 1990). Some of the precursors of BaP reactive metabolites, such as the 3-hydroxy BaP and 7,8-diol, are lipophilic, resulting in an increase in uptake by plasma lipoproteins (Shu and Nichols, 1981), transported through blood, and undergo cellular internalization (Busbee *et al.*, 1990). The phase I metabolites are conjugated with glutathione, sulfate, or glucuronic acid to form phase II metabolites. The phase II metabolites (4,5-diol glucuronide; 9,10-diol glucuronide; 3(OH) glucuronide; 3(OH) sulfate; 9(OH) sulfate; 7,8-diol GSH; and 9,10-diol GSH) are more hydrophilic and hence amenable for elimination through excretion (Ramesh *et al.*, 2011). Phase II metabolism is considered a detoxification reaction. However, activation of some of the phase II metabolites cannot be ruled out because of alterations in electrophilicity as BaP undergoes simultaneous and stereoselective metabolic transformations, like many chemical carcinogens (Oesch *et al.*, 1990).

Of the drug-metabolizing enzymes that contribute to differential susceptibilities to the adverse effects of BaP, the CYP1A1 is not constitutively expressed and CYP1A2 is mostly hepatic (Guengerich, 1997). Therefore, the biotransformation of BaP in extrahepatic tissues such as testis or ovary may have been the result of CYP1B1, which is constitutively expressed in these tissues (Otto *et al.*, 1992). The biotransformation enzymes that metabolize PAHs such as CYP1A1, CYP1A2, CYP1B1, UDP glucuronyltransferase, and glutathione S-transferase have been reported to be constitutively expressed in livers of goats, sheep, and pigs (Kaddouri *et al.*, 1990; Eltom and Schwark, 1999; Kojima *et al.*, 2008).



Our understanding of potential health risks of PAHs to livestock is based on laboratory rodent studies. A majority of these studies have been conducted with the sole aim of understanding the risks to human health, especially cancer. The relevance of these data to livestock health is a matter of debate and presents a major lacuna in regard to veterinary PAH toxicology. Collection of robust and statistically reliable data from target tissues requires terminal surgeries on multiple days after exposure. Although routinely followed in laboratory animal models, this strategy of sacrificing a considerable number of farm or domesticated animals per species raises ethical questions from an animal welfare standpoint. This is a major factor that limits our ability to pursue PAH disposition studies in livestock/domesticated animals. To overcome this limitation, our research group has used microsomes isolated from liver, testis, and ovary tissues of farm animals collected from abattoirs to study the metabolism of PAHs such as BaP and fluoranthene (Harris *et al.*, 2009). Microsomes are the subcellular organelles that harbor drug-metabolizing enzymes. These organelles have long been used as one of the *in vitro* models for studying the metabolism of toxic chemicals.

Microsomes were isolated from the liver and reproductive tissues of rat, mouse, boar, bull, cow, goat, and sheep and incubated with 5  $\mu$ M BaP. Post-incubation, samples were extracted with ethyl acetate and analyzed for BaP/metabolites by reverse-phase high-performance liquid chromatography with fluorescence detection (Walker *et al.*, 2006; Harris *et al.*, 2009). Our results revealed a great variation among species to metabolize BaP. Compared to rodents (rat and mouse), the BaP metabolite concentrations produced by bull/cow, goat, sheep, and pig testicular or ovarian or hepatic microsomes were higher ( $p < 0.05$ ). Hepatic microsomes produced the greatest quantities of metabolites relative to that of testicular and ovarian microsomes in all the species studied ( $p < 0.05$ ). This observation is not surprising inasmuch as the liver functions as the main detoxifying organ in mammalian species. Because liver is the principal detoxifying organ of BaP, it contributes metabolites through circulation to extrahepatic tissues under *in vivo* conditions. The continuous delivery of metabolites from the liver to the testis or ovary enhances the levels of reactive metabolites in reproductive tissues, which will ultimately have implications on the functioning of these organs.

The BaP metabolites identified in testis, ovary, and liver were BaP 9,10-diol, BaP 4,5-diol, BaP 7,8-diol, and 3-hydroxy and 9-hydroxy BaP. The rodent testicular microsomes produced a considerably higher proportion ( $p < 0.05$ ) of BaP 4,5-diol and 7,8-diol than did the other animal species (ewe, sow, and cow). However, hepatic microsomes from the latter farm animal group

converted a greater proportion ( $p < 0.05$ ) of BaP to 3- and 9-hydroxy BaP. There were no remarkable differences between hepatic and reproductive tissue microsomes of livestock in the BaP metabolite types formed. The hydroxy metabolites are believed to be a part of the detoxification pathway. However, it has been shown that 3-OH-BaP (Moorthy *et al.*, 2003) and 9-OH-BaP (Moorthy and Randerath, 1996), the products of detoxification, also react with DNA to form adducts in target tissues. Consequently, higher mammals, due to their high metabolizing capabilities, are expected to generate high concentrations of these two metabolites, whose accumulation in target tissues is relevant from a toxicological standpoint. Darwish *et al.* (2010) reported mutagenic activity of CYP1A1 in cattle, deer, and horses using BaP as a test compound. Their research showed that the ability to metabolize BaP is highest in the horse, followed by cattle and deer. These authors also demonstrated a similar trend of activities of UDP-glucuronosyltransferase in the previously mentioned species, whereas glutathione transferase activity was highest in horses, followed by deer and cattle.

### Effect of sexual maturity on BaP metabolism

To delineate the influence of sexual maturity on BaP metabolism, ovaries from commercial crossbred prepubertal gilts (7 or 8 months old; ovaries with follicles and no corpora lutea) and those of postpubertal gilts (ovaries with corpora lutea of various stages of development or corpora albicans) were used (Harris *et al.*, 2009). Inasmuch as it has been established that the metabolism of PAHs in mammalian ovary is hormonally regulated (Bengtsson *et al.*, 1987), our research group conducted a study designed to measure the BaP metabolite concentrations produced by microsomes isolated from prepubertal and sexually mature gilts and confirmed that BaP metabolism was indeed influenced by sexual maturity (Harris *et al.*, 2009). Microsomes isolated from the ovaries of sexually mature gilts that were at the mid-luteal and follicular phase of the estrous cycle metabolized BaP rapidly compared to ovarian microsomes isolated from prepubertal gilts (Table 63.4). The high concentrations of metabolites in luteal phase are probably due to a high constitutive expression of CYP in the corpora lutea (CL). Our findings are in agreement with those of Eliasson *et al.* (1997), who reported that the metabolism of dimethylbenz(a)anthracene (DMBA; a PAH compound) was highest in the CL of pigs compared to preovulatory follicles. Furthermore, the higher concentrations of BaP metabolites in the organic fraction (less polar) in the CL could be secondary to the higher lipid content of the CL.

**TABLE 63.4** Benzo(a)pyrene metabolite concentrations generated by microsomes from various stages of estrous cycle in pig

Ovarian status	Description	BaP metabolite concentrations (pmol/min/mg protein)
Prepubertal (n = 6)	Minimal estrogen stimulation of the uterus	0.10 ± 0.0001***
Prepubertal (n = 4)	Some uterine stimulation	0.12 ± 0.01**
Postpubertal (n = 2)	Follicular to late follicular phases, 14–15 CA, many 4- or 5-mm follicles	0.17 ± 0.02*
Postpubertal (n = 5)	Mid-luteal, 11–17 CL	0.22 ± 0.02

\* $p < 0.01$ ; prepubertal (some estrogen stimulation) versus postpubertal (follicular); \*\* $p < 0.001$ ; prepubertal (some estrogen stimulation) versus postpubertal (follicular and mid-luteal); \*\*\* $p < 0.0001$ ; prepubertal (minimal estrogen stimulation) versus postpubertal (follicular and mid-luteal).

CA, corpora albicans; CL, corpus luteum.

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## HEALTH EFFECTS OF PAHS IN FARM ANIMALS

There is a mistaken perception that environmental contaminants such as PAHs in farm animals are completely metabolized and excreted. The argument in support of this assumption arises from the observation that environmental levels of PAHs are low, that these compounds are extensively degraded in the gastrointestinal tract, and that the residual levels of these compounds remaining after metabolism are too low to exert any toxic effect (Rhind, 2005). On the contrary, despite the decline in the environmental release of PAHs, local inputs and different sources of pollution in the tropical belt and long-range atmospheric transport of PAHs from point source areas have led to a buildup of these compounds even in pristine areas (Ramesh *et al.*, 2011). Another related argument is that although short-term high-level exposure of farm animals to PAHs can be processed through metabolism and/or excretion of parent compounds, continuous or long-term exposure to low levels of PAHs results in bioaccumulation of persistent PAHs in target tissues (Bechtel *et al.*, 2009), and the sustained delivery of reactive metabolites from liver to vulnerable tissues/organs is sufficient to cause toxicity.

The farm environment is considerably contaminated with PAHs from feed ingredients such as dried grass and corn silage, which also registered high levels of PAHs. Using the feed PAH residue data, the calculated

intake of PAHs by cows was found to be 65–1000 times higher than the calculated intake by humans (Bulder *et al.*, 2006), which indicates a potential risk for cattle health. According to CCME (2010), the possible effects of various PAHs on exposed livestock include, but are not limited to, growth inhibition, endocrine disruption, reproductive impairment, and liver and kidney damage.

In estrogen receptor binding assays and reporter gene assays, BaP/metabolites were reported to be estrogen (Arcaro *et al.*, 1999) and androgen (Vinggard *et al.*, 2000) antagonists. Furthermore, studies conducted in our laboratory revealed that subacute exposure to BaP resulted in anti-estrogenic and anti-androgenic activities (Inyang *et al.*, 2003; Ramesh and Archibong *et al.*, 2011) that are driven by BaP metabolism. Interestingly, the microsomal CYP family of enzymes that generate these metabolites in the liver and extrahepatic tissues such as testis are also involved in steroid hormone metabolism (Jefcoate *et al.*, 2000). Upon prolonged exposure to BaP, sequestration of this chemical in high-density lipoproteins, which are essential for steroid hormone biosynthesis in the reproductive tissues, cannot be ruled out (Polyakov *et al.*, 1996). This can lead to reduced testosterone synthesis and release by the Leydig cells, with adverse implications on testosterone-regulated spermatogenesis in male farm animals.

## Reproductive and developmental toxicity

Livestock are exposed to a myriad of toxins (poisonous plants and mycotoxins) and toxicants (environmental pollutants such as pesticides, metals, PAHs, PCBs, and dioxins), both in the farm environment and in controlled settings (Archibong and Abdelgadir, 2000). In the face of threat from these chemicals and biotoxins, maintaining optimum reproductive efficiency of livestock is a constant challenge as the economic returns are affected. From this perspective, it is important to know how PAHs perturb the reproductive efficiency of farm animals/livestock. To date, no studies have been conducted on PAH-induced perturbations in fertility of farm animals. As mentioned previously, most of the studies on the endocrine disruptive actions of PAHs have been conducted using rodent models exposed to single PAH compounds for short durations, a treatise of which has been published by our research group (Hood *et al.*, 2011; Ramesh and Archibong, 2011). The laboratory rodent models differ from the bovine, ovine, or porcine animals in dietary habits, metabolism, and physiology. Nonetheless, PAHs including BaP have steric resemblance to steroid molecules (Santodonato, 1997) and have been demonstrated to have both estrogenic and anti-estrogenic activities (Chaloupka *et al.*, 1992; Charles *et al.*, 2000). Studies conducted by our research team using a rat model have

revealed that BaP affects the serum concentrations of progesterone, androgens, estrogens, prolactin, and, indirectly, luteinizing hormone (Ramesh and Archibong, 2011). Therefore, PAHs can influence reproductive efficiency in exposed farm animals in a similar manner as recorded for lower mammals, albeit to a different extent.

The adverse effect of BaP on male fertility indices was confirmed when adult male rats were exposed to BaP via inhalation. Specifically, a subacute exposure (10 days) of adult F-344 male rats via inhalation to BaP (75 µg BaP/m<sup>3</sup>) caused a significant reduction in stored sperm motility and circulating testosterone concentrations (Inyang *et al.*, 2003). Furthermore, a subchronic exposure of adult F-344 male rats to this PAH significantly reduced testis weight, spermatid numbers, stored sperm density, progressive motility, spermatozoa with normal morphology, intratesticular testosterone concentrations, and circulating testosterone (Ramesh and Archibong, 2011). The fertility indices described previously and the functioning of the male reproductive system in general are regulated by testosterone, whose synthesis and release by the testis is repressed by BaP. The mechanism by which BaP perturbs testosterone synthesis and release is linked to ROS-induced oxidative aging (Senft *et al.*, 2002) of the Leydig cells by damaging critical components of the steroidogenic pathway in the Leydig cells (Peltola *et al.*, 1996).

Sex differences in the constitutive expression of hepatic CYP1A1 and 1A2 in Meishan pigs have been reported by Kojima *et al.* (2008). These findings suggest androgen-dependent down-regulation of genes coding for the previously mentioned biotransformation enzymes. Further studies by this research group demonstrated that physiological concentrations of serum testosterone regulate the constitutive gene expression of CYP1A1 and 1A2 in Meishan and Landrace pigs (Kojima *et al.*, 2010). Hence, the metabolism of PAHs by the CYP family of enzymes in steroidogenic and reproductive tissues of farm animals is detrimental to hormonally regulated developmental events such as male and female gamete production, their ability to interact, and the development of generated embryo to a viable young.

The ovotoxicity of BaP has been studied in rodents. DMBA, 3-methylcholanthrene (3-MC), and BaP were reported to destroy oocytes in ovarian follicles of rats and mice (Borman *et al.*, 2000). In general, PAH parent compounds are not directly ovotoxic. They require metabolic biotransformation to reactive metabolites. Ovarian enzymes involved in the biotransformation of PAHs (CYP1B1, AHH, and epoxide hydrolase) have been identified in mice, rats, and primates (Hoyer, 2004). Consequently, oocyte destruction by PAHs may be preceded by the distribution of the parent compound to the ovary, where PAH biotransformation enzymes metabolize the compounds to reactive intermediates (Harris *et al.*, 2009). These reactive intermediates are capable of

covalent binding to macromolecules such as DNA, RNA, and proteins (Ramesh *et al.*, 2010).

Polycyclic hydrocarbons have toxic effects on mammalian fetal development. Pregnant mice or rats treated during early or middle gestation with BaP, 3-MC, or DMBA show significant increases in embryo lethality and resorption; surviving fetuses have a greater incidence of malformation (Galloway *et al.*, 1980). Our findings in F-344 rats indicate that transplacental exposure to BaP affects fetal survival, intrauterine growth, and pregnancy-related hormones (Ramesh and Archibong, 2011). Fowler *et al.* (2008) showed that low doses of environmental chemicals present in sewage sludge significantly perturb fetal ovarian development in sheep. A marked decline in prolactin, estradiol concentrations, and follicle numbers was observed. Also, an alteration in balance between pro- and anti-apoptotic proteins and a reduced level of superoxide dismutase expression were noted. These findings emphasize that long-term exposure to low doses of complex environmental mixtures such as sewage sludge (of which PAHs are a component; Smith *et al.*, 2001) may result in impaired fertility of livestock.

Reports indicate that PAHs interact with the endoplasmic reticulum (ER)  $\alpha$  and  $\beta$  signaling pathways in a variety of test systems and subsequently interfere with estrogen signaling in important reproductive processes (Arcaro *et al.*, 1999; Fertuck *et al.*, 2001). Exposure to PAHs has been shown to suppress estrogen response element-controlled gene expression and increase estradiol metabolism and down-regulation of ER  $\alpha$  levels (Safe, 2001). The action of PAHs on the steroidogenic activities in the ovary is not limited to inhibition of estrogen production. It also enhances the clearance of circulating estrogens. Induction of CYP1A1 in porcine ovarian granulosa cells by 3-MC indicates that PAHs may decrease plasma estrogens by enhancing their clearance through CYP1A1 induction (Leighton *et al.*, 1995). The P450 isoforms CYP1A1, CYP1A2, and CYP1B1 are involved in PAH biotransformation (Ramesh *et al.*, 2004). Induction of P450s by PAHs may also lead to increased metabolism of estrogens because the same P450 isoforms are involved in estrogen catabolism. For example, estrogens are metabolized by CYP1A1, CYP1B1, CYP3A4, and CYP3A7 to catechol estrogens. The catechols undergo metabolic redox cycling to generate free radicals such as reactive quinone/semiquinone intermediates (Jefcoate *et al.*, 2000). These metabolic intermediates initiate toxicity. Therefore, it can be concluded that the repression of estrogen biosynthesis and enhanced metabolism of E<sub>2</sub> by BaP can result in the reduction of the circulating concentrations of this ovarian steroid below physiological threshold essential for maintaining reproductive health.

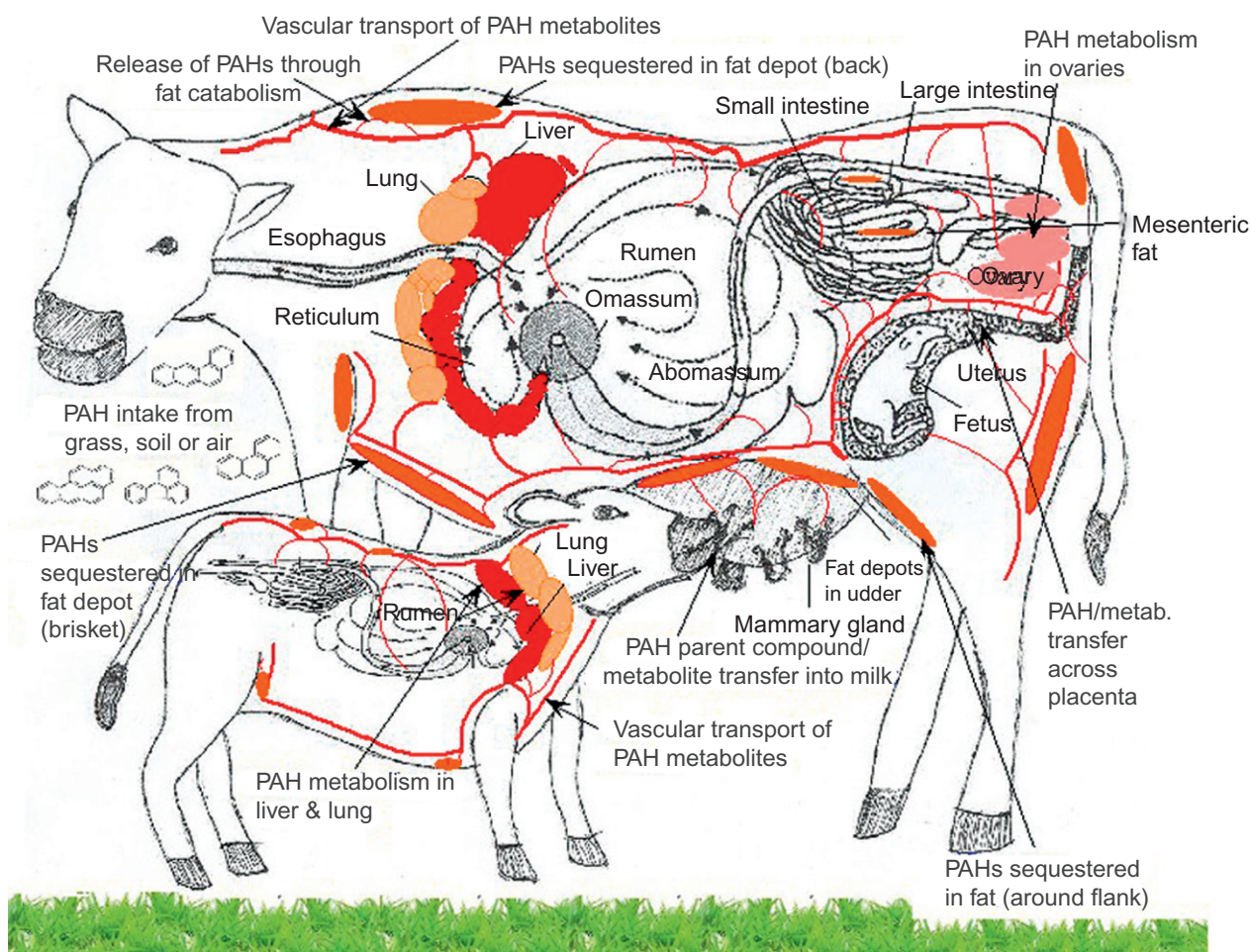
Because PAHs are lipophilic, they reside in fat depots and tissue lipids. In addition to mesenteric, pericardial, and perirenal fat, most farm animals deposit



subcutaneous fat in loin, ribs, tail head, brisket (lower part of the chest), flank, around ovaries and lipid components of the gonads, and in udder (mammary gland). These sites are potential storage depots for PAHs. In a continuous exposure scenario, the more lipophilic and high-molecular-weight PAHs tend to bioaccumulate to a greater extent in lipid-rich tissues and lipid depots. These persistent isomers are not easily available for metabolism. Even if some of these PAH parent compounds are partially metabolized, the reactive metabolites generated are released into the bloodstream. Busbee *et al.* (1990) demonstrated that gastric instillation of BaP in sheep was transported to vascular circulation in association with lipoproteins, and this absorbed fraction was internalized in vulnerable tissues. This situation ensures insults at the cellular level, culminating in organ

dysfunction. In addition, conditions such as malnutrition and aging mobilize fat reserves in the body. As a result of fat catabolism, the previously sequestered PAHs are released directly into the bloodstream, thus bypassing the hepatic detoxification. Compared to lower mammals, livestock such as cattle, sheep, goats, pigs, and camels enjoy a long life span. Hence, the consequences of long-term low-level exposures to PAHs via feed, soil, and inhalation could be deleterious not only to the farm animals per se but also for the survival and propagation of the previously mentioned species. The metabolic fate of PAHs in a pregnant cow and suckling calf are shown in Figure 63.2.

There is little information available to establish an exposure–effect relationship pertaining to PAH exposure, incidence of pathological abnormalities, and



**FIGURE 63.2** Exposure pathways, disposition, and metabolic fate of PAHs in farm animals. Upon intake of PAHs by livestock through feed, these compounds are metabolized in the intestine, liver, and reproductive tissues. The metabolites are carried to other organs and tissues of the adult animals through circulation and to pre- and postnatal animals through placenta and milk, respectively. Some of the high-molecular-weight PAHs and/or their metabolites are sequestered in fat depots in various parts of the body. Catabolism of these lipid pools releases the PAH parent compounds and/or their metabolites into circulation, contributing to total body burden of these toxicants. Because some of these metabolites are estrogenic and/or anti-estrogenic, they interfere with hormonally regulated events such as sexual maturity, reproduction, and embryo development.



incidence of cancer in animals of agricultural and veterinary interest. However, the incidence of gastric and mammary cancers in wildlife (Martineau *et al.*, 2002) and humans who inhabit areas that are in proximity to aluminum smelters (Bye *et al.*, 1998) does not rule out the possible involvement of PAHs in causing such effects in farm animals too. Given the ability of PAHs to suppress the immune system (Davila *et al.*, 1996), a survey was conducted by Bechtel *et al.* (2009) to determine whether the immunity of cattle was compromised due to PAH exposure. Blood samples were collected from yearling cattle on farms located near oil and natural gas facilities. These animals were also exposed to particle-bound PAHs released from flares. The B lymphocytes, populations of T lymphocyte subtypes, and antigen-specific serum antibodies were enumerated along with particulate matter that was less than 1  $\mu\text{m}$  in diameter. The lack of consistency in results indicates no significant association between exposures to PAH and immune system functions (Bechtel *et al.*, 2009). Whether the cattle were exposed to PAH concentrations that were high and stable enough to cause immunological adverse effects is open for speculation. Perhaps PAHs may not be sufficiently strong immunosuppressants in livestock to cause cancerous tumors but may exert toxicity to other organ systems.

## TREATMENT

Specific antagonists or antidotes to counter PAH-induced toxicity in livestock are not available. Skin contamination may be reduced by thorough scrubbing with detergents and water. For fur-bearing animals such as sheep, shearing is required before washing. Personnel involved in cleaning animals that live near oil and natural gas facilities (rich sources of PAHs) must wear personal protective equipment such as safety glasses, gloves, masks, and aprons to prevent self-contamination.

Although oral administration of charcoal is a decontamination strategy that may be used for other toxicants, it may not be a preferable one given the fact that the palatability of charcoal is very poor. Also, charcoal may contain residual levels of PAHs that may exacerbate toxicity. Rationing of feed intake is another time-tested strategy used for decontaminating the lipophilic toxicants. The idea behind this strategy is that a reduction in body fat may release the PAHs from the fat depots. Although this strategy may yield results for male and nonpregnant female livestock, it may not be practical for lactating animals because they eliminate the PAHs through milk secretion. Furthermore, rationing of feed to dams may impact the well-being of calves because of malnutrition.

If the animals are raised for milk or meat production, farmers may be reluctant to adopt this strategy. Another effective strategy is to feed the animals some herbage or silage or hay that is rich in phytochemicals or phytoestrogens because most of these phytochemicals have high detoxification potential and chemopreventive properties. Legumes, peanuts, broccoli, and soy may be supplemented in feed. The efficacy of these measures can be tested by monitoring the levels of PAHs in blood, urine, and manure (if possible) subsequent to administration of these bioactive feed supplements. These remedial measures coupled with a proactive background environmental monitoring (checking for PAH residue levels in air, soil, feed, etc.) for PAH contamination could reduce exposure to PAHs.

## CONCLUSION AND FUTURE DIRECTIONS

Despite the attention PAHs have commanded during the past several decades in view of their environmental prevalence and contamination of animal-derived products, there is inadequate information regarding the toxicity of these compounds to livestock. The uptake, accumulation, and persistence of PAHs in livestock tissues and their transfer to food products can have an impact on human health.

The internal dose determines the chemical behavior and manifestation of symptoms of toxicity in livestock. In other words, the net absorption is the critical factor that controls the internal dose. Some ingredients in livestock feed contain fat, and to increase the yield of meat and for greater economic returns, farmers tend to use fatteners and feed supplements for feedlots. Because PAHs are absorbed concomitantly with fat, consumption of PAH-contaminated fatteners may result in carryover of PAHs from feed ingredients to edible products (milk, meat, and eggs). However, studies thus far have focused on bioaccessibility of PAHs given through hay, soil, or milk but not commercial feed and additives or supplements. Thus, more work needs to be done on the absorption of PAHs taken through diverse matrices for modeling the chemical fate of ingested PAHs with the ultimate purpose of establishing any species specificity. The stability of metabolic products of PAHs in edible products requires attention. Although the metabolites of more lipophilic PAHs may not undergo carryover (to milk) compared to their less lipophilic counterparts, the stability of these PAH metabolites in food products derived from animals that have been subjected to frequent episodes of PAH exposure is open for speculation. Also, the interactions between PAHs and metals, PAHs

and veterinary drugs, and PAHs and pesticides in livestock are not yet known. In the environment, PAHs can react with pollutants such as nitrogen oxides and chlorine, yielding nitro- and chlorinated PAHs, respectively. These compounds are more persistent than unsubstituted PAH congeners and are likely to contaminate pastures and soil. Due to various interactions such as additivity, synergism, antagonism, and inhibition that occur in real-world chemical mixtures, information on the carryover will be of use in undertaking risk management practices.

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# Brominated flame retardants and perfluorinated chemicals

Prasada Rao S. Kodavanti and Bommanna G. Loganathan

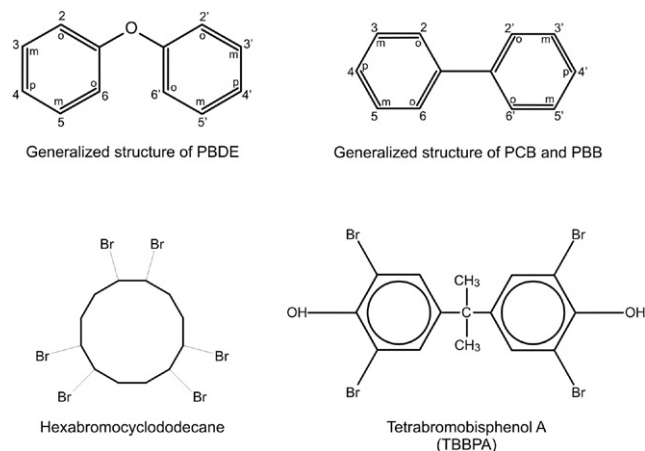
## INTRODUCTION

During the past several decades, concern has been raised regarding exposure and harmful health effects of synthetic organohalogen compounds, especially chlorine-containing compounds such as chlorinated pesticides and polychlorinated biphenyls (PCBs) in domestic animals, wildlife, and humans (Kodavanti *et al.*, 2008; Loganathan and Masunaga, 2008). There is mounting evidence that bromine- and fluorine-containing organohalogens are emerging as new pollutants that pose a threat on the global scale for present and future adverse health effects in animals and humans (Shaw and Kannan, 2009; Shaw *et al.*, 2010; Tanabe and Minh, 2010). Polybrominated diphenyl ethers (PBDEs) constitute an important group of chemicals of brominated flame retardants (BFRs) predominantly used in numerous industrial and consumer products to make these materials more fire resistant (Alaee *et al.*, 2003; Blum, 2010). The general chemical formula for PBDEs is  $C_{12}H_{10-x}Br_xO$ , and molecular weight ranges from 233 to 943. Perfluorinated compounds (PFCs) such as perfluorooctane sulfonate/sulfonic acid (PFOS) and related compounds are used as surfactants and have a wide range of industrial and commercial applications. The chemical formula for PFOS is  $C_8HF_{17}O_3S$ , and its molecular weight is 500. The structures of selected PBDEs and PFCs are shown in Figures 64.1–64.3. Unlike chlorinated compounds such as DDT (1,1,1-trichloro-2,2-bis[*p*-chlorophenyl]-ethane) and other pesticides (hexachlorobenzene, hexachlorocyclohexanes, etc.) that are used as agricultural/public health

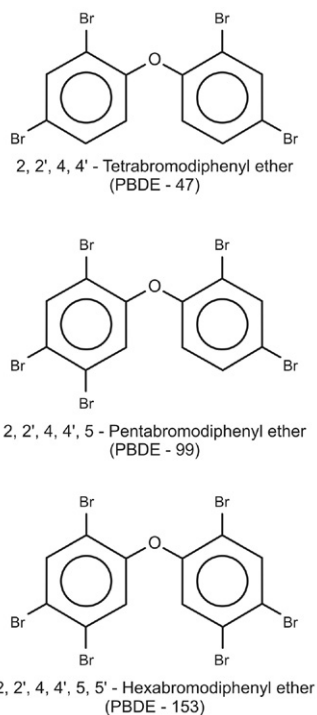
insecticides, use of BFRs and/or PFCs has never resulted in direct exposure to farm and/or domestic animals and pet animals. However, due to their unique physicochemical and biochemical properties, both BFRs and PFCs persist and pervade every component of the global ecosystem, leading to exposure to animals and humans and contributing to negative health effects (Blum, 2010; Loganathan, 2011). The Stockholm Convention on persistent organic pollutants (POPs) included some of the PBDEs and PFCs in the POPs list (Stockholm Convention Secretariat, 2010). Although the production and use of PBDEs and PFCs are restricted, environmental contamination and harmful effects to animals and humans continue. In this chapter, the historical background of BFRs and PFCs, physicochemical properties, exposure, pharmacokinetics, mechanism of action, and effects are discussed with emphasis on domestic and pet animals.

## BACKGROUND

The first incidence of accidental poisoning caused by brominated compounds occurred in the early 1970s. Inadvertent use of polybrominated biphenyls (PBBs sold under the trade name FireMaster) in place of a cattle feed supplement, magnesium oxide (sold under the trade name NutriMaster), in the production of feed for dairy cattle resulted in the destruction of approximately 1.5 million chickens, 5 million eggs, 29,800 cattle, 34,000 pounds of milk products, 5920 hogs, and 1470 sheep on



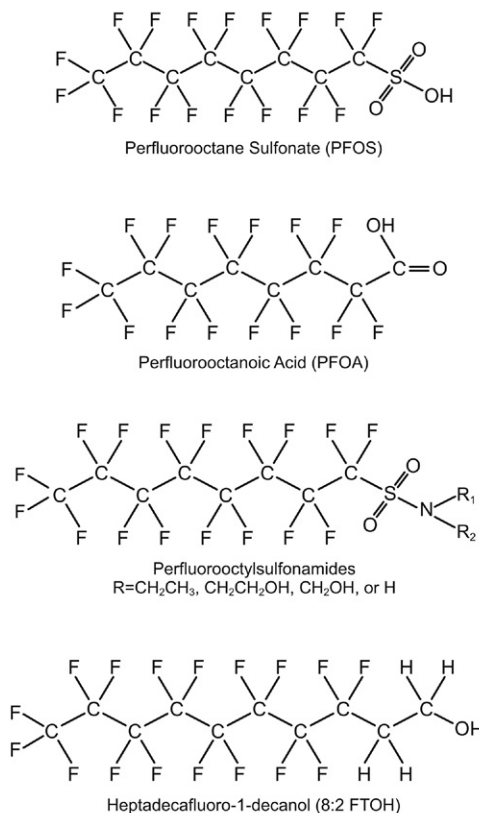
**FIGURE 64.1** Chemical structures of brominated flame retardants. Structural similarities between polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs) are shown that are relevant to their mode of action. The letters “o,” “m,” and “p” respectively indicate *ortho*, *meta*, and *para* substitutions for chlorines or bromines. The numbers indicate the positions of halogens.



**FIGURE 64.2** Chemical structures of predominant PBDEs found in commercial mixtures as well as biological and environmental samples.

Michigan farms. The cost of this damage was approximately \$75–\$100 million (Carter, 1976; Fries, 1985). After this incidental poisoning via cattle feed, PBBs (Figure 64.1) were removed from the market. Of the brominated compounds still on the market, brominated bisphenols,

#### Chemical Structures of Selected Perfluorinated Compounds



**FIGURE 64.3** Chemical structures of predominant perfluorinated chemicals, PFOS (perfluorooctane sulfonic acid) and PFOA (perfluorooctanoic acid). The metabolites perfluorooctylsulfonamide and heptadecafluoro-1-decanol are shown for structural comparison.

diphenyl ethers, and cyclododecanes are three major classes that are still produced in large volumes (Shaw and Kannan, 2009). It is estimated that more than 1 million metric tons of PBDEs have been produced. Although the manufacture of penta- and octa-bromo mixtures has ceased, the production of decabromodiphenyl ether (decaBDE) continues in some countries (Guo *et al.*, 2011). DecaBDE was banned in Sweden and followed by partial bans in four U.S. states (Washington, Maine, Oregon, and Vermont) in 2007, the European Union in 2008, and Canada in 2009. The REACH (Registration, Evaluation, Authorization, and Restriction of Chemical Substances) program in the European Union announced in February 2011 the ban of hexabromocyclododecane (HBCD) used with all polystyrene in building insulation ([http://ec.europa.eu/environment/chemicals/reach/reach\\_intro.htm](http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm)). In general, PBDEs and other brominated and chlorinated flame retardants in use are HBCD, decabromodiphenyl ether, tetrabromobisphenol-A (TBBPA), tris(1-chloro-2-propyl)phosphate,

tris(2-chloroethyl)phosphate, and Dechlorane Plus. These compounds are used in furniture, textiles, polyurethane foam, plastics used in electric and electronic equipment, printed circuit boards, curtains, carpets, etc. to meet fire safety standards (Alaee *et al.*, 2003; Stapleton *et al.*, 2009; De Wit *et al.*, 2010; Guo *et al.*, 2011). The high production volume and the structural similarities of these brominated chemicals to other well-known toxic environmental contaminants such as DDTs and PCBs are the main concerns for environmental and human/animal health. Furthermore, polybrominated dioxins (PBDDs)/dibenzofurans (PBDFs), formed during heating or incineration of BFRs, have toxicological profiles similar to those of their chlorinated homologs (Birnbaum *et al.*, 2003; DiGangi *et al.*, 2010), but they are more toxic than PBDEs. Like other organohalogenes, BFRs are ubiquitous in the environment, bioaccumulate, and are toxic to animals and humans (Dye *et al.*, 2007; Kodavanti *et al.*, 2008; Kierkegaard *et al.*, 2009; Ounnas *et al.*, 2010; Shaw *et al.*, 2010; Guo *et al.*, 2011).

PFCs are another class of persistent organohalogenes. PFCs differ from brominated and chlorinated organohalogenes by virtue of all hydrogen atoms, except those in the functional groups being substituted by fluorine atoms (Figure 64.3). PFCs have been used in a variety of specialized consumer and industrial products for more than 60 years. The applications of PFCs include heat-, chemical-, and abrasion-resistive coatings on utensils; they are also used as dispersion, wetting, or water protection for paper and surface treatments. In particular, PFCs are used in metal-plating baths, surfactants, cleaning products, rust inhibitors, fire-fighting applications, starting materials for polymers, herbicide and insecticide formulations, cosmetics, shampoos, pharmaceuticals, lubricants, paints, polishes, upholstery, textiles, carpets, soil/stain resistance coatings, mining and oil well surfactants, acid mist suppressants, electronic etching baths, alkaline cleaners, floor polishes, photographic film, and denture cleaners and adhesives (Senthilkumar, 2005; Kodavanti *et al.*, 2008; Yamashita *et al.*, 2011). PFCs are also used in paper protection, including food contact applications (plates, food containers, bags, and wraps) and non-food contact applications (folding cartons, carbonless forms, and masking papers) (Kannan *et al.*, 2002).

As a result of widespread use of these compounds, concern regarding PFC contamination has increased. PFOS and perfluorooctanoic acid (PFOA) have been consistently detected in environmental matrices, animals, and human tissues (Kannan *et al.*, 2004, 2005a,b). Although PFOCs have been produced since the late 1950s, these compounds were first reported to occur on a global scale in 2001 (Giesy and Kannan, 2001). Perfluorinated contaminants, such as perfluorobutanesulfonate, perfluorohexanesulfonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic

acid (PFUnDA), perfluorododecanoic acid (PFDOA), and perfluorooctanesulfonamide (PFOSA), are less frequently detected in biota than are PFOS and PFOA. Studies suggest that PFCs accumulate in farm and pet animals such as chicken, cattle, goats, horses, pigs, and other vertebrates (Kennedy *et al.*, 2004; Guruge *et al.*, 2008).

## PHYSICOCHEMICAL PROPERTIES

### Brominated flame retardants

TBBPA (Figure 64.1), the highest volume flame retardant worldwide, is primarily a reactive BFR (90%) covalently bound to the polymer structure and less likely to be released into the environment than are additive flame retardants (Birnbaum and Bergman, 2010). TBBPA is used in several types of circuit board polymers. TBBPA is highly lipophilic ( $\log K_{ow} = 4.5$ ) and has low water solubility (0.72 mg/mL). TBBPA has been measured in the air (Zweidinger *et al.*, 1979), soil, and sediment (Watanabe *et al.*, 1983), but it is generally not found in water samples. TBBPA is found in eggs of birds, human milk, and umbilical cord serum. TBBPA derivatives such as ethers are reported to be biologically active, which may lead to health effects (Legler, 2008; Birnbaum and Bergman, 2010). HBCD (Figure 64.1) is a nonaromatic brominated cyclic alkane with a molecular weight of 641.7, and it is mainly used as an additive flame retardant in thermoplastic polymers with final applications in styrene resins (National Research Council, 2000). Like other BFRs, HBCD is highly lipophilic, with a  $\log K_{ow}$  of 5.6, and it has low water solubility (0.0034 mg/L) (MacGregor and Nixon, 1997). The melting point is 185–195°C, and vapor pressure is  $4.7 \times 10^{-7}$  mmHg. Studies have shown that HBCD is highly persistent, with a half-life of 3 days in air and 2025 days in water (Lyman, 1990), and it is bioaccumulative, with a bioconcentration factor of approximately 18,100 in fathead minnows (Veith and Defoe, 1979).

PBDEs are composed of two phenyl rings linked by an oxygen (thus the designation as “ether”; Figures 64.1 and 64.2). The phenyl rings may have 1–10 bromine atoms, leading to the formation of 209 possible congeners. The exact identity and pattern of various congeners in various commercial mixtures depend on the manufacturer and the specific product. Among these, the commercial “penta” mixture generally consists of PBDE congeners 99 (pentaBDE) and 47 (tetraBDE) as the major constituents (Figure 64.2), which comprise approximately 70% of the mixture (Huber and Ballschmiter, 2001). PBDE congener 100 (pentaBDE) is present at less than 10%, with PBDE congeners 153 and 154 (hexaBDEs) at less than 5% each. The commercial “octa” mixture is 10–12% hexaBDE, 43–44% heptaBDE, 31–35% octaBDE, 9–11%

nonaBDE, and 0–1% decaBDE. The “deca” commercial mixture consists of 98% decaBDE, with a small percentage of nonaBDEs (World Health Organization, 1994; LaGuardia *et al.*, 2006).

PBDEs are structurally similar to PCBs (Figure 64.1) and elicit adverse effects similar to those of PCBs on nervous, immune, and endocrine systems. They also influence metabolism of chemicals endogenous to the body as well as that of foreign chemicals. Compared to chlorine atoms, bromine atoms are in general lost more easily from the molecule (more “reactive”), rendering PBDEs more susceptible to various types of degradation and metabolism than PCBs (Table 64.1). Trace analysis of these commercial mixtures for other brominated contaminants revealed the presence of PBBs and PBDFs, but not PBDDs, at levels above the limit of detection (Hanari *et al.*, 2006). It is known that PBDEs, upon pyrolysis at 900°C, release

PBDFs and PBDDs, and the amount of these contaminants depends on the conditions of pyrolysis (Buser, 1986; Thoma *et al.*, 1986; Shaw *et al.*, 2010).

## Perfluorinated compounds

Perfluorinated compounds have ionic and neutral characters. Because of their thermodynamically strong covalent carbon–fluorine bonds, these compounds were initially considered as nontoxic and nonmetabolizable. It is apparent that carbon–fluorine bond has maximum energy compared to other bonds. Most of them were also considered to have little volatility. PFCs with considerable volatility are fluorotelomer alcohols (FTOHs or hydroxylated PFCs), which together with some sulfonamide-based PFCs may undergo transformation in the environment to form more persistent PFOA or similar perfluoroalkyl carboxylic or sulfonic acids. PFCs are water soluble in the several parts per million range (Table 64.2). PFCs with unique surface modification properties readily bind to surfaces including blood globulins (Kodavanti *et al.*, 2008).

The stability of the carbon–fluorine bond contributes to the persistence of PFCs in the environment and their ability to bioaccumulate and biomagnify in higher trophic levels of a food chain. PFOS has been shown to bioconcentrate from water into benthic invertebrates by three orders of magnitude and to bioaccumulate in top predators by 5- to 20-fold (Kannan *et al.*, 2005a). PFCs are also detected in wildlife and human tissues. Bald eagles, river otters, and bottlenose dolphins accumulate PFOS in their livers at microgram per gram tissue concentrations

TABLE 64.1 Physical and chemical properties of polybrominated diphenyl ethers

Property	PBDEs (penta-, octa-, and deca-BDE)
Physical state at room temperature	Pale yellow liquid to white powder
Molecular weight	564–959.2
Water solubility (µg/L at 25°C)	1
Boiling point (°C)	< 300 to >400
Melting point (°C)	85–306
Vapor pressure at 25°C (mmHg)	$2.2 \times 10^{-7}$ to $9 \times 10^{-10}$
Log $K_{ow}$	5.7–8.27
Henry's law constant (atm m <sup>3</sup> /mol)	$7.5 \times 10^{-8}$ to $1.2 \times 10^{-5}$

Data from ATSDR (2004) and De Wit (2002).

TABLE 64.2 Physical and chemical properties of perfluorinated compounds

Compound	Boiling point (°C)	Melting point (°C)	Vapor pressure at 20°C (Pa)	Water solubility (mg/L)	pK <sub>a</sub>	Henry's law constant (atm m <sup>3</sup> mol <sup>-1</sup> )
Perfluorooctane sulfonyl fluoride (POSF)	154–155		221			
Perfluorooctane sulfonic acid (PFOS)	149	70–100				
Perfluorooctane sulfonate potassium salt (PFOS K)		<400	$3.31 \times 10^{-4}$	570		$7.2 \times 10^{-9}$
N-ethyl-perfluorooctanesulfonamide (N-EtFOSA)	~110	~90	0.16			
Perfluorobutanoic acid (PFBA)	120	–19.5	1333			
Perfluoropentanoic acid (PFPeA)	127					
Perfluorohexanoic acid (PFHxA)	157	12–14				
Perfluoroheptanoic acid (PFHpA)	175–177					
Perfluorooctanoic acid (PFOA)	189–192	55	100	3400	2.5	$4.6 \times 10^{-6}$
Perfluorononanoic acid (PFNA)		71–77				
Perfluorodecanoic acid (PFDA)	218	83–85				
Perfluoroundecanoic acid (PFUnDA)	160	96–101				
Perfluorododecanoic acid (PFDoDA)	245	107–109				
8:2 fluorotelomer alcohol (8:2 FTOH)	95–105	42–44	356 at 25°C	0.14		$9.6 \times 10^{-2}$

Data from U.S. EPA (2000), Giesy and Kannan (2001), and Yamashita *et al.* (2011).



(Senthilkumar, 2005; Houde *et al.*, 2006). Furthermore, fluorinated acids are even found in ocean waters, Arctic ice, and Antarctic environment and biota (Yamashita *et al.*, 2011). Physical and chemical properties of PFCs are summarized in Table 64.2. PFCs are ubiquitous in the environment and bioaccumulate in animals and humans; their toxic properties are being revealed (Gill *et al.*, 2004).

## PHARMACOKINETICS AND TOXICOKINETICS

### Brominated flame retardants

The major pathway of TBBPA exposure is through diet, despite its low levels in the average diet in the United States. The bioavailability of TBBPA in circulation is very low even after administration of a high dose to rats due to rapid metabolism, indicating its low potential to bioaccumulate and cause toxic effects (Shaw *et al.*, 2010). TBBPA or its metabolites such as TBBPA-glucuronide, a diglucuronide, and a mixed glucuronide-sulfate were detected in bile and were excreted predominantly in feces; however, metabolites were also found in urine (Hakk *et al.*, 2000). After intravenous injection into rats, TBBPA was also rapidly cleared, and the majority of the intravenous dose was also recovered in the feces (Kuester *et al.*, 2007). Kinetic studies in rodents and humans suggest low absorption of TBBPA from the gastrointestinal tract and rapid metabolism of the absorbed TBBPA by conjugation (Schauer *et al.*, 2006; Kuester *et al.*, 2007).

Information on the toxicokinetics of HBCD is limited. HBCD is absorbed from the gastrointestinal tract, and major sources of human exposure are food and dust intake. There are three main diastereoisomers in the commercial HBCD mixture, denoted as  $\alpha$ ,  $\beta$ , and  $\gamma$ , with the  $\gamma$ -diastereoisomer predominating (>70%) (Heeb *et al.*, 2005). High levels of HBCD in some top predators indicate the persistence and biomagnification of HBCD. However, most early studies did not examine individual HBCD diastereoisomers but only the commercial HBCD mixture. Recent studies have shown that there is a predominance of the most persistent stereoisomer,  $\alpha$ -HBCD, in biota (Covaci *et al.*, 2006; Law *et al.*, 2006) and that individual diastereoisomers have different physical and chemical properties. There is a growing need to understand the individual diastereoisomers that make up the commercial mixture.

There are significant differences in the pharmacokinetic behavior of individual PBDE congeners and mixtures. These differences in absorption, distribution, metabolism, and excretion depend on the test animal species and the degree of bromination. As the number of bromine atoms increases from 4 to 10 bromines (tetraBDE to decaBDE), there are decreases in oral absorption, which leads to

shortened half-lives and increased elimination in both urine and feces. DecaBDE is poorly absorbed, with greater than 90% of the dose excreted in feces within 48 h (Morck *et al.*, 2003). Workers occupationally exposed to decaBDEs have shown decaBDE half-lives of approximately 15 days (Thuresson *et al.*, 2006), which is considerably shorter than those of other lower brominated congeners. DecaBDE has been found in the blood and breast milk of humans in the general population, but at lower levels than other PBDE congeners (Lorber, 2008).

Lower brominated PBDEs, in contrast to decaBDE, are more readily absorbed and persist in the body for many years because they are mainly stored in body fat. PBDE-47 and PBDE-99 are well absorbed and highly distributed to lipophilic tissues, such as adipose, skin, and liver tissue (Hakk *et al.*, 2002; Staskal *et al.*, 2005). In rats, tetra- and pentaBDEs are metabolized and eliminated slowly (Hakk *et al.*, 2002), whereas in mice, PBDE-47 is well absorbed and distributed, but its elimination is also rapid (Staskal *et al.*, 2005). Uptake efficiencies of BDE-47, BDE-99, and BDE-153 by pike fed trout injected with the congeners were 90, 62, and 40%, respectively (Bureau *et al.*, 1997).

### Perfluorinated compounds

Very limited information is available on the pharmacokinetics and toxicokinetics of perfluorochemicals in farm or domestic animals. Laboratory animal studies have shown that PFOS and PFOA are well absorbed orally, not metabolized, and poorly eliminated (Ophaug and Singer, 1980; DuPont Company, 1982; Johnson *et al.*, 1984; Vanden Heuvel *et al.*, 1991; Guruge *et al.*, 2006). Upon ingestion, these compounds are distributed primarily to the liver, kidney, and serum, with liver concentrations being the highest (Seacat *et al.*, 2002, 2003; Hundley *et al.*, 2006). PFOS and PFOA tend to bind to  $\beta$ -lipoproteins (Jones *et al.*, 2003), albumin, and liver fatty acid binding protein (Luebker *et al.*, 2002). Elimination rates and half-lives of PFCs vary with carbon chain length and animal species and gender tested. Hanhijarvi *et al.* (1988) reported that male hamsters excrete PFOA more rapidly than do females. In dogs, the half-life of PFOA is 20–30 days in males and 8–13 days in female. However, gender differences are not discernible in mice or rabbits (Hundley *et al.*, 2006; Lau *et al.*, 2006). The cause for gender and species differences in elimination of PFCs is not elucidated. Buist and Klaassen (2004) attributed these differences to the functions of organic anion transporters in the kidney because several transporter proteins are expressed differentially in male and female adult rats. Due to gender and species differences in elimination of PFCs, careful consideration must be given to compare toxicological effects. Further studies with emphasis on body burden rather than administered dose are warranted.

## MECHANISM OF ACTION AND TOXICITY

### Brominated flame retardants

TBBPA is of high ecotoxicologic concern due to its acute and chronic toxicity in several biota (U.S. EPA, 2008). TBBPA has been shown to be rapidly metabolized by mammalian livers and eliminated in bile, urine, and feces (Schauer *et al.*, 2006). TBBPA has been detected in various environmental media and biota, including air, soil, water, sediment, and bird muscle from electronic waste processing regions of China (Shi *et al.*, 2009), and also in bottlenose dolphins and bull sharks from the Florida coast (Johnson-Restrepo *et al.*, 2008). Currently, no restrictions are placed on the production and use of TBBPA. TBBPA is a cytotoxicant, immunotoxicant, and thyroid hormone (TH) agonist, and it has the potential to disrupt estrogen signaling (Birnbaum and Staskal, 2004). TBBPA is also toxic in rat brain cells *in vitro*, where it causes oxidative stress, calcium influx, and inhibits dopamine uptake (Reistad *et al.*, 2007). *In vivo* studies indicate that neonatal TBBPA exposure causes hearing deficits in rat offspring, similar to those observed following developmental exposure to PCBs (Lilienthal *et al.*, 2008). Nakajima *et al.* (2009) reported the presence of TBBPA in brain along with behavioral alterations following acute treatment of TBBPA. Disruption of TH homeostasis is proposed to be the primary toxic effect of TBBPA and other BFRs. TBBPA has a closer structural relation to thyroxine ( $T_4$ ) than to PCBs and binds to transthyretin (TTR) with greater affinity than it does to  $T_4$  (Meerts *et al.*, 2000). The detailed mechanism by which BFRs can disrupt TH homeostasis is discussed later (Figure 64.4).

Experimental studies showed that HBCD, the second most used flame retardant, has negative effects on endocrine and reproductive processes (Birnbaum and Staskal, 2004). Many effects of HBCDs seem to occur during development. During developmental exposure, HBCDs have been shown to decrease bone density and retinoids and to enhance immune response to sheep red blood cells (van der Ven *et al.*, 2009). HBCD isomers are endocrine disruptors with anti-androgenic properties that inhibit aromatase and interact with steroid hormone receptors (Hamers *et al.*, 2006). Like other BFRs, HBCDs may disrupt TH homeostasis, resulting in decreased  $T_4$  levels and increased thyroid-stimulating hormone (TSH) (Ema *et al.*, 2007). Studies indicate that a low dose of HBCD can potentially disrupt TH hormone receptor-mediated transactivation and impairs cerebellar Purkinje cell dendritogenesis (Ibhazehiebo *et al.*, 2011). The detailed mechanism by which BFRs can disrupt TH homeostasis is discussed later (Figure 64.4).

PBDE residues have been detected in indoor air, house dust, and foods (Schechter *et al.*, 2006; Sjodin *et al.*, 2008).

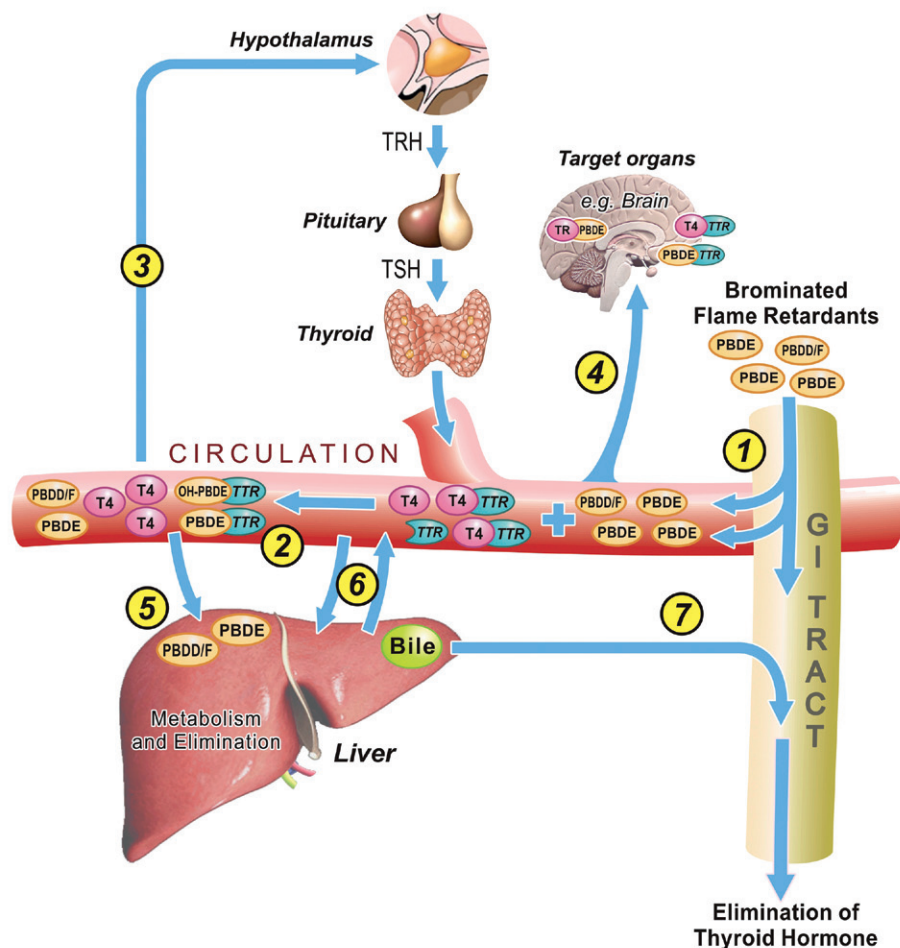
Sewage treatment plant effluents and biosolids are considered a major source of PBDEs (Rieck, 2004). More than half of the sewage sludge produced annually in the United States is applied to land as fertilizer (U.S. EPA, 1999). Agricultural land that has been treated with sewage sludge can be highly contaminated with PBDEs. Thus, application of sewage sludge may represent a source of exposure by direct contact or uptake through plants. In grazing animals including cattle, soil ingestion leads to contamination of meat and dairy products. In dairy cattle, the major intake of organohalogenes occurs via spontaneous soil ingestion during grazing (Fries, 1995). Cows ingest up to 1000g of soil per day (i.e., several micrograms of halogenated compounds daily) depending on several factors, including season, climate, and density of grass (Laurent *et al.*, 2005).

There are a few reports indicating high levels of serum PBDEs in household cats because of their high exposure to house dust (Dye *et al.*, 2007). In captive giant and red pandas, the tissue levels of PBDEs ranged from 38 to 2158ng/g lipid weight (lw) (Hu *et al.*, 2008). Kierkegaard *et al.* (2009) reported PBDE levels in cow milk (1100–2600pg/g lw) and fat (1300–2600pg/g lw). In Arctic fox, the levels of PBDEs are very low, ranging from 26 to 31ng/g lw (De Wit *et al.*, 2010). Whereas the levels of PBDEs in the liver of California sea otter are 2423ng/g lw (Kannan *et al.*, 2008), the levels are much higher in blubber of California sea lions, ranging from 569 to 24343ng/g lw (Stapleton *et al.*, 2006). In adipose tissue of polar bears in Canada, Muir *et al.* (2006) reported levels of PBDEs ranging from 4.6 to 11ng/g lw, and these levels were lower than those reported from polar bear fat samples from Svalbard and East Greenland. PBDE levels in domestic, pet, and wild animals are presented in Table 64.3.

Exposure to PBDEs in domestic/pet animals and humans may occur via multiple sources (air, water, dust, and food). Levels of PBDEs in animal and human tissues have increased exponentially since the 1970s in several countries, including the United States, Canada, and Sweden (Schechter *et al.*, 2005; Guo *et al.*, 2011). Elevated levels of PBDEs in North America have been attributed to the greater use of the pentaBDE mixture compared to its use throughout the rest of the world. Like other lipophilic compounds, PBDEs readily cross the placenta into the fetus, providing an opportunity for PBDEs to interfere with human and animal developmental processes.

Because PBDEs are predominantly used indoors, data on their effects on wildlife and farm animals are limited. A few studies indicate that PBDE exposure at environmentally relevant concentrations increases nestling growth (Fernie *et al.*, 2006) and causes changes in reproductive courtship behaviors in adult American kestrels (Fernie *et al.*, 2008). However, a number of studies

## Possible Mode(s) of Action for Thyroid Hormone Disruption



**FIGURE 64.4** Possible mechanisms by which BFRs can disrupt thyroid hormone (TH) homeostasis. PBDE is shown as an example of all BFRs. (1) BFRs as well as their contaminants enter the circulation from the gastrointestinal (GI) tract. (2) BFRs (parent compound as well as metabolites) can displace thyroxine (T4) from serum binding proteins such as transthyretin (TTR). The resulting free T4 will be subjected to hepatic metabolism and elimination. (3) Reduced circulating T4 levels trigger the hypothalamic-pituitary axis to synthesize and secrete more T4 by thyroid. (4) BFRs bound to TTR along with T4 will reach target organs including brain, where they can bind to TH receptor to elicit a biological/toxicological response. (5) After entering liver activate nuclear receptors, BFRs initiate transcription of xenobiotic-metabolizing enzymes for T4 metabolism and elimination. (6) Influx transporters further increase the T4 uptake and metabolism. (7) Efflux transporters eliminate T4 or its conjugates from liver into either serum or the bile. Figure is not to scale. Adapted from Kodavanti and Curras-Collazo (2010).

on laboratory animals have indicated that commercial PBDE mixtures as well as the individual PBDE congeners that compose them affect the nervous, endocrine, reproductive, and immune systems. With regard to neurotoxic effects, several studies have indicated that HBCD and PBDEs cause permanent aberrations in spontaneous behavior and habituation capability in mice after a single exposure at postnatal day (PND) 10 (a period of rapid brain growth development). It is interesting to note that the effects seen on this behavioral paradigm with PBDEs are identical to those produced by PCBs (Eriksson and

Fredriksson, 1996). Mice exposed to a single dose of PBDE 47 on PND 10 demonstrated delayed ontogeny of neuromotor function and hyperactivity when they attained adult age without any alterations in circulating TH levels (Gee and Moser, 2008; Gee *et al.*, 2008). Other studies showed developmental delays in the acquisition of the palpebral reflex following neonatal exposure to PBDE 209 along with changes in circulating T<sub>4</sub> levels (Rice *et al.*, 2007). Because of these differential reports, the role of hypothyroxinemia with regard to the behavioral effects of PBDEs is unclear. However, the mechanisms

TABLE 64.3 Polybrominated diphenylether levels in domestic, pet, and wild animals

Animal	Sample	Total PBDE concentration	Unit	Age (years)	Reference
Household cat <sup>a</sup>	Serum	4.3 ± 1.5 (3.5)	ng/MI	1.25–5	Dye <i>et al.</i> (2007)
	Serum	10.5 ± 3.5 (5.9)	ng/mL	8–15	Dye <i>et al.</i> (2007)
	Serum	12.7 ± 3.9 (6.2)	ng/mL	10–18	Dye <i>et al.</i> (2007)
Captive giant and red panda	Tissue	38.4–2,158	ng/g lw	NA	Hu <i>et al.</i> (2008)
Cow	Fat tissue	1,300–2,700	pg/g lw	NA	Kierkegaard <i>et al.</i> (2009)
Cow	Milk	1,100–2,600	pg/g lw	NA	Kierkegaard <i>et al.</i> (2009)
Moose	Liver	0.24–26 (BDE-47)	ng/g lw		De Wit <i>et al.</i> (2010)
		0.26–34 (BDE-99)			
Arctic fox	Subcutaneous adipose tissue	26.3	ng/g lw	NA	De Wit <i>et al.</i> (2010)
	Abdominal adipose tissue	31.6	ng/g lw	NA	De Wit <i>et al.</i> (2010)
	Blubber	569–24,343	ng/g lw	NA	Stapleton <i>et al.</i> (2006)
Polar bear	Adipose tissue	4.6–11	ng/g lw		Muir <i>et al.</i> (2006)
California sea otter	Liver	2,423 ( <i>n</i> = 6)	ng/g lw		Kannan <i>et al.</i> (2008)
Silver perch	Whole fish	337 ( <i>n</i> = 2)	ng/g lw		Sajwan <i>et al.</i> (2008)

<sup>a</sup>Total PBDE concentrations in parentheses indicate median.

NA, not available.

by which PBDEs or other BFRs disrupt TH homeostasis are shown in Figure 64.4. BFRs as well as their contaminants enter the circulatory system from the gastrointestinal tract. T<sub>4</sub> is synthesized and released into circulation by the thyroid gland. In circulation, BFRs (parent compound as well as metabolites) can displace T<sub>4</sub> from serum binding proteins such as TTR. The resulting free T<sub>4</sub> will be subjected to hepatic metabolism and elimination. The reductions in circulating T<sub>4</sub> levels increase TSH production via reduced negative feedback on the hypothalamic–pituitary axis, which induces increased synthesis and secretion of T<sub>4</sub> by the thyroid gland. BFRs bound to TTR along with T<sub>4</sub> will reach target organs including the brain, in which they can bind to the TH receptor to elicit a biological/toxicological response. In the liver, BFRs activate nuclear receptors, initiating transcription of xenobiotic metabolizing enzymes for T<sub>4</sub> metabolism and elimination. Influx transporters further increase the T<sub>4</sub> uptake and metabolism, whereas efflux transporters eliminate T<sub>4</sub> or its conjugates from the liver into either serum or the bile (Figure 64.4). Given the key role of TH in development, BFR-induced perturbations in TH homeostasis might play a role in the outcome of adverse effects associated with BFR exposure.

Studies indicate that a commercial pentabrominated mixture (DE-71), when administered during development, did not alter maternal or male offspring body weights. However, female offspring were smaller compared to controls from PND 35 to 60. DE-71 exposure also accumulated PBDE congeners in various tissues including brain, suggesting that PBDEs cross the blood–placenta and blood–brain barriers, causing subtle changes in some parameters of neurobehavior and

dramatic changes in circulating thyroid hormone levels, as well as changes in both male and female reproductive end points (Kodavanti *et al.*, 2010).

In addition to the effects on TH, there is evidence that PBDEs affect the cholinergic neurotransmitter system (Viberg *et al.*, 2003a,b), which is involved in memory and motor function, among others. Several PBDE congeners have been compared to PCBs with regard to their ability to affect intracellular signaling in a cerebellar (brain) culture system (Kodavanti and Ward, 2005; Kodavanti *et al.*, 2005; Fan *et al.*, 2010). The Ca/protein kinase C signaling pathways are also proposed as mechanisms of neurotoxicity for a number of chemicals, including PCBs and PBDEs. The order of potency for their effects on intracellular signaling was DE-71 (a commercial mixture of tetra-, penta-, and hexaBDEs) > 47 > 100 > 99. On a molar basis, DE-71 was equipotent with Aroclor 1254; the most widely used commercial PCB mixture. A Swedish study found that PBDE-99 and PCB-52 produced effects on behavior when given together but not at the same dose given alone (Eriksson *et al.*, 2006). These results suggest that there may be little difference in neurotoxic potency between PBDEs and PCBs, and that effects of PCBs and PBDEs are additive. This implies that body burdens of PCBs and PBDEs in humans may need to be added when assessing risk.

## Perfluorinated compounds

Table 64.4 summarizes PFC concentrations in farm and pet/captive animals. Detectable levels of PFCs were found in serum samples from chicken, pigs, cattle (Jack Black),



TABLE 64.4 Perfluorinated compound concentrations in farm and pet/captive animals

Species	N	Location	PFC concentrations (ng/mL wet wt.)					Reference
			PFOS	PFHxS	PFDA	PFNA	PFOA	
Chicken/serum	7	Japan	4.9	0.07	0.06	0.22	0.44	Guruge <i>et al.</i> (2008)
Pig/serum	6	Japan	0.57	0.04	0.07	0.12	0.23	Guruge <i>et al.</i> (2008)
Cattle, Jack Black/serum	5	Japan	1.5	0.01	0.48	0.04	0.01	Guruge <i>et al.</i> (2008)
Horse/serum	2	Japan	0.82	<0.01	0.5	0.04	<0.05	Guruge <i>et al.</i> (2008)
Dog/serum	10	Japan	25	10	0.28	1.5	2.5	Guruge <i>et al.</i> (2008)
Goat/plasma	5	Japan	2.4	0.05	1.0	1.2	0.13	Guruge <i>et al.</i> (2008)
Amur tiger/serum	100	China	1.41	0.11	0.07	0.32	0.11	Li <i>et al.</i> (2008)
Polar bear/liver <sup>a</sup>	NR	Svalbard	1290	2940	43	102	21	Kannan <i>et al.</i> (2005b)
Giant panda/serum	9	China	11.10	NA	NA	NA	0.80	Dai <i>et al.</i> (2006)
Red panda/serum	12	China	15.65	NA	NA	NA	2.29	Dai <i>et al.</i> (2006)
Bottlenose dolphin/serum	2	Bermuda	49	5.9	9.6	17	0.8	Giesy and Kannan (2001)

<sup>a</sup>Indicates levels found in liver compared to others reported in serum.

NA, not available.

horses, dogs, and goats from Japan (Guruge *et al.*, 2008). Among several PFCs measured, PFOS and PFOA concentrations were consistently higher in almost all samples. The serum PFOS and PFOA concentrations ranged from 0.57 to 4.9 ng/g wet weight and from less than 0.05 to 2.5 ng/g wet weight, respectively (Table 64.4). Serum samples of Amur tigers, giant pandas, and red pandas from China also contained detectable concentrations of PFCs (Dai *et al.*, 2006; Li *et al.*, 2008). As observed in farm animals, Amur tiger and panda serum samples had comparatively higher concentrations of PFOS than other PFCs, including PFOA. Animal tissue samples from remote locations, such as polar bear liver samples from Svalbard and serum samples from bottlenose dolphin from Bermuda, also showed relatively high concentrations of PFOS compared to other PFCs (Table 64.4). Polar bear liver contained 1290 ng/g wet weight of PFOS and 2940 ng/g wet weight of PFHxS (Kannan *et al.*, 2005b).

Perfluorochemicals, especially PFOS and PFOA, were associated with liver enlargement and hepatocellular adenomas in rats (Lau *et al.*, 2007). Lau and co-workers suggested that agonism of the peroxisome proliferator-activated receptor- $\alpha$  (PPAR- $\alpha$ ) may be involved in tumor (primarily liver) induction by a number of non-genotoxic carcinogens in the rodents. Following this, a number of studies were conducted to determine whether the PPAR- $\alpha$  agonistic mode of action is involved in the liver toxicity and hepatocellular adenomas observed in rats treated with PFOS and PFOA. Several short-term studies in rats and mice have revealed that PFOS and PFOA are capable of inducing peroxisome proliferation, and the first key event in this mode of action is activation of PPAR- $\alpha$  (Ikeda *et al.*, 1987; Berthiaume and Wallace, 2002; 3M Company, 2004; Vanden Huevel *et al.*, 2006). Because several PFCs can activate PPAR- $\alpha$ , these

compounds may also induce peroxisome proliferation by perturbing lipid metabolism and transport. Maloney and Waxman (1999) reported that PPAR- $\alpha$  was activated by endogenous cellular fatty acids. Based on these observations, Lau *et al.* (2007) suggested that displacement of endogenous ligands from L-FABP may be one of the mechanisms by which PFOS induces peroxisome proliferation. In addition, Yang *et al.* (2002a,b) showed that the activation of PPAR- $\alpha$  by PFOA was consistent with a significant increase in liver weight in wild-type mice exposed to dietary PFOA or WY-14,643, a classical peroxisome proliferator, at 0.02 and 0.125% (wt/wt), respectively, for 7 days.

Because PPAR- $\alpha$  controls lipid metabolism and transport, the hepatomegaly observed in PPAR- $\alpha$  in null mice was attributed to accumulation of lipid droplets or accumulation of PFOA in the liver (Yang *et al.*, 2006). Guruge *et al.* (2006) studied gene expression profiles in rat liver with PFOA and showed that the largest categories of induced genes are those participating in metabolism and transport of lipids (fatty acids). PFOA not only exhibits properties of a peroxisome proliferator but also possesses the properties of a mixed-type enzyme-inducing agent, as demonstrated by marked induction of CYP2B2, CYP3A4, and CYP4A1 in liver microsomes (Elcombe *et al.*, 2007). Kudo *et al.* (2000) studied the induction of peroxisomal  $\beta$ -oxidation by perfluorochemicals in rat livers by administration of PFCs at doses ranging from 2.5 to 20 mg/kg/day for 5 days and found that in male rats, all compounds except PFHA induced the activity of peroxisomal  $\beta$ -oxidation. However, in female rats, PFNA and PFDA induced the activity. Differences in accumulation of these compounds in the rat livers may be responsible for the different hepatic responses observed between various PFCs with different carbon chain length and between sexes.

In multicellular organisms, cells communicate (exchange ions, send messages, and exchange small molecules) by a process known as gap junctional intercellular communication (GJIC). GJIC maintains tissue homeostasis and is involved in growth, development, and differentiation (Lau *et al.*, 2007). Trosko and Rush (1998) suggested that loss of GJIC plays a role in carcinogenesis. Hu *et al.* (2002) showed that rats exposed orally (5 mg/kg) for 3 days or 3 weeks inhibit GJIC, and GJIC is also inhibited in a dose-dependent manner in rat liver and dolphin kidney cell lines. However, the pathophysiological significance of GJIC inhibition with regard to the carcinogenic mode of action for PFOS and PFOA is unclear.

It is important to mention that PBDEs, PFOS, and PFOA have strong potential for reproductive and developmental toxicity (Johansson *et al.*, 2009; Kodavanti *et al.*, 2011; Abdelouahab *et al.*, 2011).

## CONCLUSION AND FUTURE DIRECTIONS

Brominated flame retardants and perfluorinated compounds are considered emerging environmental pollutants. During the past few decades, there has been a great deal of progress in understanding the distribution of these pollutants in the global environment, wildlife, and humans. Although monitoring studies have clearly shown the presence of BFRs and PFCs worldwide, environmental/biological transformation and toxicology, pathways, and mode of action are not fully understood or described. Furthermore, contamination levels and potential negative effects of these toxic pollutants on domestic and pet animals have been largely ignored. Laboratory animal studies and accidental poisoning case studies have shown that exposure to BFRs and/or PFCs may result in serious health effects, including endocrine disruption leading to reproductive and immune dysfunction, birth defects, neurotoxicity, and certain types of cancers. Based on laboratory and field study results, environmental exposure of farm animals to BFRs and PFCs is of great concern because these compounds can affect animal health as well as serve as a source for human exposure via consumption of contaminated meat and/or dairy products. Although some BFRs have been banned or voluntarily withdrawn by manufacturers, human/animal exposure to these chemicals will continue for a long time, as is the case with chlorinated organic chemicals that exist in large quantities in consumer products (Shaw *et al.*, 2010). Therefore, research is needed to address the long-term consequences of exposure to these chemicals even as new brominated replacement chemicals enter into the market.

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# Veterinary geology

Jan G. Myburgh

## INTRODUCTION

Links between the natural environment and human health have been recognized for centuries (Webb, 1964; Plant *et al.*, 1996; Skinner 2007). Ancient Chinese, Egyptian, Islamic, and Greek writings described many health problems. Hippocrates and Aristotle noted relationships between environmental factors and the distribution of various diseases (Bunnell, 2004). However, it is not only areas with excesses (toxicity) that are important; extensive regions have also been shown to have levels of essential inorganic elements well below (deficiency) what is needed (Plant *et al.*, 1996). The positive effects of a large number of different minerals that were used for medicinal purposes were already described more than 2000 years ago in Chinese texts, and these were used for their healing properties (Bunnell, 2004).

Similarly, environmental effects on animal and plant health are also well recognized (Underwood and Suttle, 1999; Whitehead, 2000; He *et al.*, 2005). Farm animals and wildlife are in general more directly dependent on their local environment for the supply of inorganic elements than are humans (Thornton, 2002, 2010; Steinnes, 2009). Animals are seldom moved around, except if they belong to nomadic farmers, and are constantly exposed to local environmental factors (geochemistry and water) if abnormal. Humans, on the other hand, usually buy vegetables and food at local markets that have come from other areas, even in developing countries, thereby effectively reducing the risk of being affected by local anomalies in the area (Mills, 1996; Plant *et al.*, 1996).

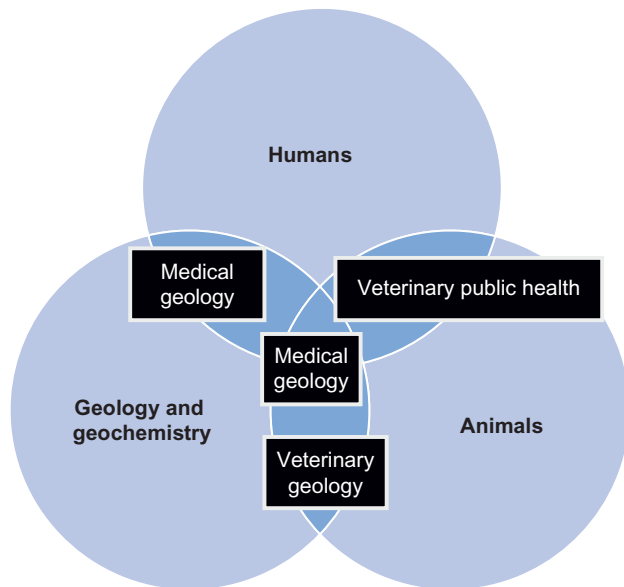
Because livestock and wildlife are closely associated with their environment, any geological anomaly (abnormality in soil chemistry and groundwater

composition) will be mirrored in their health (Edmunds and Smedley, 1996; Plant *et al.*, 1996; Thornton, 2002; Frank, 2004). Animals that are looked after intensively, such as dairy cows and feedlot animals, receive their feed from other areas and are unlikely to be affected by a local problem, except for abnormalities in groundwater (e.g., fluorosis). Wildlife migration probably reduces the risk of exposure to local geochemical anomalies (Thornton, 2002; Maskall and Thornton, 1996).

## MEDICAL GEOLOGY

Medical geology is a rapidly growing field concerned with the relationship between natural geological factors and human and animal health (Selinus *et al.*, 2005). To support this multidisciplinary field, the International Medical Geology Association ([www.medicalgeology.org](http://www.medicalgeology.org)) was formed, and a textbook (*Essentials of Medical Geology*) was published by prominent researchers in this field (Davies *et al.*, 2005). Several other textbooks and numerous publications have been published (Bowman *et al.*, 2003; Bunnell, 2004; Bunnell *et al.*, 2007; Skinner, 2007; Dissanayake and Chandrajith, 2009; Steinnes, 2009).

Medical geology is recognized as a subject discipline, and it is taught at several universities as a specific subject (Davies *et al.*, 2005). Although it refers to animal health, the focus is mainly on human health (Aggett and Mills, 1996; Davies, 2008; Lar and Tejan, 2008; Dissanayake and Chandrajith, 2009; Rapant *et al.*, 2009). It is important to realize that animal health also affects human health through animal products such as meat and milk.



**FIGURE 65.1** Schematic relationship between human health, animal health, and environment (geology and geochemistry). The interfaces – namely medical geology, veterinary geology, and veterinary public health – are also indicated.

## VETERINARY GEOLOGY

Although the term veterinary geology has not been used before, the focus is very similar to that of medical geology (Davies *et al.*, 2005; Jones, 2005). It is proposed that we use the term veterinary geology in specific cases to emphasize the importance of geology (geochemistry and groundwater) in animal health and where animals are specifically affected or studied (Figure 65.1).

If animals are affected by their environment, very often humans living in that specific area will also be affected (e.g., fluorosis). This is very often seen in rural villages, where humans and their animals are not moving around much and must use the same environment and water. Medical doctors or researchers who focus on human health usually underestimate farm animals as sentinels for environmental problems.

Veterinary geology focuses on the effects that geology and geochemistry have on the health, production, and reproduction of animals, including livestock, wildlife, and companion animals. The natural geologic environment as well as anthropogenic changes to the environment, such as mining or farming activities, should also be included. It is important to note that even drinking water may be influenced to a certain degree by the local geology (Edmunds and Smedley, 1996).

Farm animals, kept extensively or semi-extensively, and wildlife are closely associated with their natural environment. Nowadays, livestock are kept in smaller

areas due to space constraints. Geological anomalies may have a larger impact if animals are fenced in or “trapped” in these specific areas. This is more important in developing countries, where balanced rations and supplementary feeding are not as routinely used to improve the health of livestock.

In southern Africa, very expensive wildlife, such as roan, sable, and African buffalo, are also kept in small camps to prevent poaching and to facilitate constant observation. If these animals are trapped on geological anomalies, it will affect their health, especially if they are kept in one of these areas for long periods. Wildlife farmers very often believe that wildlife do not need special attention, as required by livestock; however, if they are fenced in, in a specific area, they need to be managed like farm animals. Farming with wildlife outside their normal distribution ranges has also caused problems due to animals not adapted to specific areas. Animals take in soil while grazing, and this may also influence their health in either positive or negative ways (Kreulen, 1985).

## PROBLEMS IN ANIMALS ASSOCIATED WITH GEOCHEMISTRY

Rural people have always recognized that the health of their animals is influenced by the animals’ diet and, therefore, soil properties. These observations could not be explained until the advent of scientific agriculture in the 19th century, when it required only a small step to suggest that humans may also be caught up in similar relationships. Diseases now known to be caused by a lack or excess of elements in the soil and plants were given names that reflected where they occurred, such as Derbyshire neck in the iodine-deficient areas of the English Midlands or Bodmin Moor sickness over the granites of southwest England, where cobalt deficiency is endemic in sheep unless treated. It is interesting to note that in Japan, before the 1868 Meiji Restoration, meat was rarely eaten so there was no tradition of animal husbandry. Japanese authors have suggested that this lack of animal indicators largely contributed to the failure to recognize the significance of metal pollution until it became catastrophic (Davies *et al.*, 2005).

It is important that veterinarians, animal scientists, and soil scientists take note of veterinary geology. Plants will also to some degree reflect what is in the environment. Geological anomalies may affect the health of animals through mineral imbalances (e.g., high molybdenum and copper deficiency), deficiencies (e.g., low cobalt in sandy soil), or intake of abnormally high concentrations of elements (e.g., selenium) (Frank, 2004; Plant *et al.*, 1996; Thornton, 2002; Underwood and Suttle, 1999).



Livestock and wildlife sometimes deliberately eat soil. However, the involuntary intake of soil through normal grazing probably influences the mineral status of animals more than does the deliberate eating of soil. Geophagia is well known but still not clearly understood (Kreulen, 1985).

In Southern Africa very expensive wildlife (roan, sable and buffalo) are often kept in smaller camps. If these animals are trapped in an area with a geological anomaly, their health will be affected.

During the purchase of a farm, it is imperative that a scientist examine the local geology and advise the prospective buyer about the environment. Migration of animals is not possible anymore in the modern farming community. Nomadic farming with cattle is disappearing, and the natural migration of wildlife is hampered by fences, highways, and private property. Animals moving into an area low seasonally could take in high concentrations of specific elements. However, very often they move out at the end of the season and are not clinically affected. Modern humans buy from local markets, thus avoiding problems because products are delivered from different areas (Maskall and Thornton, 1996; Thornton, 2002).

Mining has a major effect on animal health. Anthropogenic changes to the geological environment are also included in this definition. The veterinary public health issue must also not be ignored.

## PROBLEMS ASSOCIATED WITH WATER

Whoever wishes to investigate medicine properly, should proceed thus.... We must also consider the qualities of the waters, for as they differ from one another in taste and weight, so also do they differ much in their quality.

Hippocrates (460–377 BC)

The detrimental effects of poor quality drinking water are well known (Edmunds and Smedley, 1996; Dissanayake and Chandrajith, 2009). Poor quality water may cause acute deaths (e.g., nitrate poisoning) or chronic health effects (e.g., fluorosis) in farm animals. It is possible to delineate large areas containing element deficiencies that are closely related to the local geology and/or geographical location. Three elements (Se, Fe, and I) cause well-documented deficiency-related health problems, although Se and Fe also give rise to disease if presented above threshold values (Edmunds and Smedley, 1996).

Water, however, may be a useful indicator of local environmental levels of trace elements (e.g., in food, soil, rocks, and the atmosphere). The link between excesses or deficiencies of particular trace elements and the health of

humans and animals are likely to be more noticeable in developing countries, especially in rural areas, because of a much greater dependence on water and food of local provenance (Edmunds and Smedley, 1996; Fordyce *et al.*, 1996; Plant *et al.*, 1996).

## CONCLUSIONS

Because livestock and wildlife are closely associated with their environment, especially animals that are kept extensively, any geological anomaly (abnormality in soil chemistry and groundwater composition) will be mirrored in their health. Although the term veterinary geology has not been used before, it is very similar to medical geology, with a focus on animal health. The term veterinary geology is proposed to emphasize the importance of geology in animal health and to be used when referring to where animals are specifically affected or studied.

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# Principles of ecotoxicology

Val R. Beasley and Jeffrey M. Levensgood

## INTRODUCTION AND CORE CONCEPTS

One Health is a term that is gaining ground because it recognizes the reality that the health of humans, domestic animals, wildlife, and ecosystems are inextricably interdependent. One Toxicology is a term reflecting similar interdependence in the study of chemically induced diseases and how human toxicology, domestic animal toxicology, wildlife toxicology, and ecotoxicology inform one another (Beasley, 2009). Also, poorer people and wildlife have much in common. They tend to be exposed to highly toxic chemicals in the environment more than their wealthier or domesticated counterparts. Poor people and wildlife also rely more on native species for food; therefore, protecting one group can often protect the other. This chapter provides information on select toxicants and issues of importance in ecotoxicology and environmental toxicology (i.e., the study of outdoor contaminants on human beings).

### Definitions

Hoffman *et al.* (2003) defined ecotoxicology as “the science of predicting effects of potentially toxic agents on natural ecosystems and on non-target species.” Newman (1998) offered a more expansive definition of ecotoxicology as the “science of contaminants in the biosphere and their effects on constituents of the biosphere,” but this could be taken to encompass all of toxicology. Of course, ecology is the science of the interactions among living organisms and the environment, and ecotoxicology includes not only predictions but also diagnoses,

management, and preventive actions. Although ecotoxicology draws on knowledge from all species, it focuses on free-ranging life-forms. We define toxicology as the science of the adverse biochemically mediated effects of all chemicals on all life-forms. Thus, we define ecotoxicology as the science of the adverse biochemically mediated effects of all chemicals and combinations of chemicals on all free-ranging organisms and on their interactions with one another and the environment. The breadth of ecotoxicology is apparent not only in these definitions but also through common types of research that it involves, which include studies of (1) the transport, fate, and concentrations of contaminants in the environment; (2) the fate of contaminants in native species; (3) the pathophysiologic effects of single chemicals on a single native or surrogate microbe, plant, or animal species in the laboratory; (4) the pathophysiologic effects of controlled amounts of single chemicals and groups of chemicals on suites of organisms in microcosms or mesocosms; and (5) the impacts of environmental contaminants on suites of biotic and abiotic components in field studies. Because of the permutations of organisms and exposures in the world today, ecotoxicology is clearly one of the most encompassing and challenging specialties under the larger umbrella of toxicology.

### Successes and challenges

The necessity of integrated field and laboratory research was illustrated in studies of dichlorodiphenyltrichloroethane (DDT) on avian reproduction. The insecticidal properties of DDT were discovered during World War II, and it was used to counter malaria and typhus in troops via control of mosquito and flea vectors,

respectively. Agricultural applications began after World War II. Large amounts were sprayed on farm fields in the United States, Great Britain, and elsewhere. According to the Agency for Toxic Substances and Disease Registry, DDT production peaked in 1962, when 85,000 tons were produced in the United States alone. An estimated 675,000 tons were applied in the United States during 1945–1972. Due to its low acute toxicity to most bird and mammal species, DDT was initially considered safe.

After noting increased raptor nests with broken eggs in the United Kingdom, Ratcliffe (1967) undertook research that revealed precipitous declines in ratios of shell weights to egg sizes in three species of birds from 1946 to 1950, after which the thinning persisted. Thickness of eggshells from 1947 to 1967 was significantly less than that from 1900 to 1946. Jefferies (1967) linked DDT to delayed ovulation in birds, suggesting endocrine disruption. This was followed by many corroborative field and laboratory studies that offered overwhelming evidence on the insidious, devastating impacts of DDT on avian reproduction. The effects of DDT on eggs were ultimately linked to its persistent metabolite, dichlorodiphenyldichloroethylene (*p,p'*-DDE), which inhibits prostaglandin synthetase and CarATPase, resulting in reduced calcium uptake by the shell gland mucosa during eggshell formation. Due to the thin eggshells, adult birds of many species crushed their eggs during incubation (Henny *et al.*, 1985; Burgers *et al.*, 1986; Newton *et al.*, 1989; Wiemeyer *et al.*, 1993; Bowerman *et al.*, 1995; Lundholm, 1997; Grasman *et al.*, 1998).

Pest resistance necessitating development of alternatives, decades of accumulated evidence on environmental impacts, and the publication of *Silent Spring* by Rachel Carson in 1962 prompted the ban on most uses of DDT by the U.S. Environmental Protection Agency (EPA) in June 1972. Many other developed nations banned DDT and pressured less affluent countries to discontinue or curtail its use. In 2005, however, the World Health Organization and the Stockholm Convention on Persistent Organic Pollutants condoned limited use of DDT for indoor mosquito control in tropical malaria-endemic areas.

Although DDE residues are declining, it is still widely disseminated in the environment. Also, an emerging concern regards freeing of organochlorines from melting ice caps. This phenomenon has been linked to elevated concentrations of parent DDT in Adelie penguins in the Antarctic (Geisz *et al.*, 2008).

Research and stewardship in ecotoxicology have (1) helped reduce dissemination of some major environmental pollutants (e.g., DDT, polychlorinated biphenyls (PCBs), and lead shot), (2) prompted development of chemicals of lower environmental risk (e.g., insect growth hormone mimics), (3) helped applied ecology to focus more on biochemical endpoints, (4) improved the effectiveness of ecological restorations in previously

contaminated habitats, and, thus, (5) increased the effectiveness of conservation medicine and ecosystem health professionals.

Despite the success stories of ecotoxicology, as well as conservation biology and ecosystem health, stewardship remains extremely uneven throughout the United States and the world. In many places, since the publication of *Silent Spring*, mismanagement of ecological resources has continued or even accelerated. The failure to protect groups of organisms that represent millions of years of coevolution is often related to powerful economic interests (e.g., mining, petroleum, energy generation, synthetic chemical, construction, and agricultural and forestry industries), poor people who are desperate for food and income, and short-sighted political pressure. The outcomes include tolerance of avoidable contamination that results in profound stress, impaired reproduction, and reduced survival in wild plants and animals. Of concern is that no new, major legislation in support of ecosystem health has been passed by the U.S. Congress since 1990. Increasingly, it is Europe, rather than the United States, that is becoming the proactive leader in regard to environmental contaminants.

Environmental pollutants of great importance in the 21st century include industrial chemicals, combustion products, pesticides, flame retardants, salts, acids, nutrients, and heavy metals. Combined with aggressive and careless actions that cause habitat loss, overharvest of native species, introductions of exotic species, climate change, and increasing exposures to pathogens, contaminants are contributing to an accelerating sixth extinction (Pimm *et al.*, 1995). Problems related to environmental contamination persist in much of the developed world, and they are burgeoning in areas of the developing world where mineral extraction, industrialization, and urbanization are proceeding at unprecedented rates and environmental monitoring and regulation are weak and often corrupt. The developed world increasingly relies on agricultural products, raw materials, and manufactured goods from the developing world. Unfortunately while developed world citizens may insist on ecological stewardship in their home countries, many remain oblivious to their roles as consumers whose purchases drive uncontrolled pollution of the developing world.

Problems are made worse by the limited involvement of veterinary toxicology in the generation of diagnostic criteria for use in ecotoxicology. Historically, few ecotoxicologists have linked exposures and residues in animals to clinical signs, clinical pathology changes, and lesions. Even now, regulatory criteria in ecotoxicology often fail to require characterization of residues and histopathologic lesions in test organisms. Thus, there is a need for more stringent regulations and, in the meantime, for more veterinary toxicologists and analytical toxicologists to take wildlife and ecological samples into the



laboratory for basic, applied, and forensic studies. Early diagnoses, source identification, pollution prevention, cleanups, and replacement of problem chemicals with less toxic alternatives can be the outcomes of applying the everyday approach of veterinary toxicologists to wildlife and ecosystems.

Improved stewardship of chemicals is a key component of reestablishing sustainability of life on Earth. Limiting the harm induced by chemical pollution should be the easy part of ecological stewardship, but those with the capacity to bring about improvements must find the resources and the will necessary to do a better job. The positive benefits of such actions include improved human economic well-being and less morbidity and mortality in wild species. Protection of ecosystems from the impacts of environmental contaminants can readily protect the lives of billions of wild plants and animals as well as the health of innumerable domestic animals and human beings. In addition to their roles as members of populations, biotic communities, and ecosystems, many wild species of shellfish, fish, birds, and mammals are economically important and extremely healthy sources of protein. However, for such species to serve as an efficient and safe source of food that can be shared by humans and predators of an array of other species, their populations must be sustainable and they must not present unwarranted chemical hazards. Major challenges are at hand, but the methods to improve conditions are known. The issue is whether they will be applied at local, national, international, and global scales to fuse and achieve economic, ethical, and ecological goals.

## Sources of pollution

Myriad toxic substances are released into our environment daily. Toxic agents are either deliberately manufactured (e.g., pesticides, drugs, construction chemicals, and household chemicals) or accidentally produced (e.g., byproducts in both final formulations and airborne, liquid, and solid waste streams). Inappropriate siting and operation of chemical manufacturing and waste storage facilities can lead to acute and chronic chemical contamination of groundwater, surface water, sediment, soil, air, and resident biota. Chemical and textile manufacturing plants, tanneries, refineries, smelters, chemical storage facilities, and electricity generating plants were often located near waterways for ease of transportation of raw materials and finished products, for access to large quantities of water used in manufacturing, and/or to allow for dissemination of wastes. Such siting predisposes to deliberate (sometimes permitted), accidental, and even malicious releases of toxic chemicals into aquatic ecosystems.

Environmental contamination can emanate from point sources such as industries, mines, refineries,

coal-burning power plants, or sewage treatment plants. Contaminants can also enter the environment from non-point sources, such as pesticides washed from large areas of land after precipitation, effluents from the tailpipes of myriad motor vehicles, or semivolatile pollutants that circle the globe after evaporation from the soils of agricultural and urban environments.

## Environmental fate and bioavailability of chemicals

Our environmental contamination problems are complex but controllable by limiting environmental pollution so that concentrations are not high enough to harm prokaryotic and eukaryotic life-forms beyond target sites, such as an agricultural field being treated because an arthropod pest is attacking a monoculture, thereby reducing the yield and quality of the harvest. Although the assimilative capacity of most natural environments is tremendous, responsible stewardship should always ensure that pollutants are not produced and used at rates that undermine the capacity of the environment for rapid detoxification.

Having a contaminant in the environment does not mean that all nearby organisms will have contact with it. For example, surface-dwelling animals may not have direct access to a contaminant buried under many centimeters of topsoil. However, certain organisms may be exposed by consuming earthworms or plants that bring the contaminants to the surface. In ecotoxicology, receptors generally refers to intact organisms. When pollutants and receptors co-occur, pathways of exposure, including dermal, ocular, oral (via water, diet, and grooming), inhalation, and/or gills, may be important. The bioavailability of a pollutant in the environment refers to the fraction of the substance that is absorbed across the gut, skin, or other portals to enter the bloodstream and other tissues where it can cause one or more pathophysiological reactions. Bioavailability can be influenced by the types and concentrations of toxicants in the environment, the nutritional status of the exposed organisms, the amounts of organic matter, pH, cation-exchange capacities of soils or sediments, and the presence of elements or other chemicals that antagonize uptake.

Environmental contaminants can be physically transformed into simpler, often less toxic forms, in several ways. Spontaneous oxidation, reduction, and hydrolysis reactions may be involved. By definition, photodegradation implies reduction in toxicity and, in the case of many complex molecules containing chlorine, bromine, or fluorine, can result from ultraviolet (UV)-induced dehalogenation. Although many man-made chemicals are rapidly photodegraded by sunlight, some chemicals become more toxic to organisms through

photoactivation. For example, dechlorination of hexachlorodibenzo-*p*-dioxin may produce lower chlorinated but more toxic dioxin congeners.

Biotransformation involves modifications of chemicals by physiological processes in living organisms (e.g., enzymes in the liver that catalyze hydrolysis, oxidation, reduction, or conjugation). Biotransformation occurs not only in animals and plants but also in soil microbes such as fungi and bacteria under aerobic or anerobic conditions. In vertebrates, microbes within digestive tracts can also greatly influence the biotransformation of environmental contaminants. Metabolism and conjugation of xenobiotics often reduces toxicity and enhances the ability of the animal to eliminate the agent from the body. By contrast, metabolic changes can also bioactivate chemicals to produce (by definition) metabolites that are more toxic than their parent compounds. For example, the organochlorine pesticide DDT is not itself highly toxic to birds, but its metabolite *p,p'*-DDE can cause thinning of eggshells due to disruption of calcium metabolism. Also, microbial metabolism in anaerobic sediments converts inorganic mercury to methylmercury, which is responsible for cognitive, motor, and visual-spatial disabilities, especially in the developing young of a wide array of animals including human beings. Efforts by organisms to detoxify exogenous compounds can sometimes produce temporary bioactivation manifested in reactive species such as singlet oxygen or hydroxy radicals. These free radicals or oxidants, as well as their depletion of natural antioxidants in the body (e.g., glutathione, ascorbic acid, and carotenoids), can lead to oxidative stress, damaging macromolecules, cells, and tissues. Such processes can culminate in overt tissue injury and also DNA damage leading to degenerative diseases, mutagenesis, carcinogenesis, and hereditary defects. To better understand what is happening to wildlife and ecosystems – as well as to domestic animals and humans – far more research is warranted on the fate and toxicity of mixtures of contaminants, as well as their metabolites, and their environmental degradation products.

### **Chemical disasters: ecological, economic, and societal factors**

The stewardship of chemically mediated disasters ranges from responsible and efficient to neglect and indifference. Localized disasters involving a single, acutely toxic, short-lived chemical (e.g., a volatile solvent released into a warm water body from a damaged tank car of a train) can be largely self-resolving. Ecological recovery after a chemical insult is also more likely when ecological resources (locally adapted microbes, plants, and animals) from the periphery are intact and able to recolonize the affected area; local emergency agencies

are well equipped, staffed, and responsive; funding is available from responsible parties or government to support containment, cleanup, and ecological rehabilitation; and the public is strongly engaged. Conversely, recovery is less likely when releases involve multiple long-lived chemicals spread over a wide area (e.g., complex, halogenated, higher molecular-weight wastes in a series of large lakes), ecological resources from the periphery are insufficient to support recolonization, responsible parties deny involvement, neither the responsible parties nor the governments involved put forth needed funds, and the public is largely disengaged. Accordingly, for the ecotoxicologist to be an effective member of a stewardship team, effective laws and policies and an informed and empowered public are often essential. Rather than tolerating or increasing releases of environmental pollutants that lead to downstream, downwind, or wider chemical disasters, enlightened leaders in industry and government are supporting harmonized global laws and regulations that impose industrywide stewardship so that they may produce and use chemicals responsibly without being at a competitive disadvantage.

### **ECOTOXICANTS IN CONCERT WITH OTHER MECHANISMS OF ECOSYSTEM DISEASE**

Prevention of ecotoxicological diseases is uniquely important because wild animals are less readily observed than people, domestic animals, exotic pets or zoo animals; toxic agents in ecosystems cause not only direct poisoning of observed organisms but also poisoning of other species on which they depend; treatment of free-ranging species is challenging if not impossible; and the survival of endangered species is commonly at risk.

Aquatic systems throughout the world are increasingly under stress from human activities. Wetlands, lakes, streams, rivers, and estuaries are being degraded due to hydrologic mismanagement, which directly stresses local aquatic life and may also aggravate chemical pollution problems. A vastly reduced sponge effect (which historically was provided by ubiquitous native plant debris), grading of land, hard surfaces, drainage of wetlands, stream channelization, and subsurface tiling of fields decrease groundwater recharge and increase the “flashiness” of streams, contributing to catastrophic and prolonged flooding. After significant rainfall, cropland (especially when largely barren in fall, winter, and spring), cleared forests, stockyards, lawns, roofs, roads, parking lots, demolition and construction sites, and poorly managed industrial and dump sites release pulses of complex pollutant mixtures to water bodies (Kolpin *et al.*, 2002). Such pulses

can carry high loads of nutrients, metals, salts, hydrocarbons, synthetic organic chemicals including pesticides, and combustion products into surface waters.

When wetlands and forests dry, essential carbon needed for water retention is oxidized, leaving the system as CO<sub>2</sub>. Also, without water recharge from wetlands, streams experience abnormally low flows during dry periods, which decrease dilution and thus increase concentrations of contaminants. Reduced volume in streams also results in increased water temperatures in summer, which greatly reduces the water's oxygen-holding capacity. Removal of tree canopies through logging, agriculture, and urban development further increases stream temperatures, harming aquatic species such as trout and salmon, which can survive only in cool, highly oxygenated waters.

In addition to being sources of toxicants, road and bridge construction, deforestation, agriculture, and mining erode soils and increase sedimentation of waterways. High suspended sediment loads increase turbidity, shading plants so that their primary productivity from photosynthesis and the dissolved oxygen, biomass, and structure that they provide are reduced. Excess suspended sediments in waterways can also degrade spawning habitat, produce lesions in gills, and modify animal behaviors, reducing fish productivity and disrupting fish community assemblages (Burkhead and Jelks, 2001).

Many of the world's coral reefs are being degraded by destructive fishing and collecting methods, eutrophication, and increasing water temperatures related to global climate change and especially runoff. Coral diseases have been attributed to thermal stress, viruses, bacteria, cyanobacteria, and fungi. Although the mechanisms of environmental degradation in producing coral disease remain poorly understood, the vast majority of impacted reefs in the Caribbean are near areas of human activity. For example, coral off the coast of the Netherlands Antilles where there are inputs of sewage have experienced black band disease, the lesions of which are characterized by changes in microbial communities. Understanding the complex relationships among black band and other coral diseases, their microbial co-inhabitants, and chemical contamination will undoubtedly require additional research (Green and Bruckner, 2000; Klaus *et al.*, 2007).

Many frontier areas of North America were initially converted to farms that consisted of a mosaic of small fields of vegetables, grains, and forage crops, interspersed with fencerows, woodlots, pastures, and wetlands. People learned to use crop rotations for both fertilization and pest control. By contrast, modern agricultural and forestry practices have produced vast monocultures of genetically similar plants that deplete nutrients and are susceptible to attack by plant pathogens such as fungi and herbivorous insects, triggering

applications of toxic fertilizers and pesticides, the effects of which are discussed later.

Modern agriculture and forestry, along with urbanization and suburban sprawl, have eliminated most natural habitat, leaving habitat islands in a largely inhospitable larger landscape. Biota trapped in such habitat remnants are prone to extirpation due to extreme weather, desiccation, inbreeding, competition with invasive and sometimes exotic species, starvation, predation, infectious diseases, and toxicoses. Individually and together, these forces can cause reduced breeding fitness and reproduction, as well as direct mortality. The resultant loss of species from habitat patches can culminate in local or regional extirpations and ultimately extinction events.

### **Wasting carbon, inducing climate change, and increasing toxic exposures**

Human-induced environmental contamination is not a new phenomenon; it is one that is becoming far more complex. In much of the midwestern United States prior to European settlement, wild habitats were burned by people to create lush grazing lands that could be used for hunting. This activity released not only smoke but also pulses of nutrients to local watersheds. Anthropogenic air pollution has been reported since 13th-century London, when smog from coal fires blanketed the city, and the situation worsened with the industrial revolution of the 1700s. An estimated 12,000 people died in London during the Great Smog of the winter of 1952. Today, climate change, flooding, fires, free nutrients, and low water flows are associated with increased toxicant exposures and ecotoxicological impacts. Excessive reliance on natural gas, petroleum, and coal for power generation releases a wide array of pollutants from tall stacks, potentially harming people, animals, and the environment both near and far from the source. Additional major anthropogenic sources of today's atmospheric pollutants include oil refinement, motor vehicles, waste incineration, industrial emissions, fires from slash-and-burn agriculture in previously forested areas, annual clearing of land by burning before planting of crops (common in much of the world), and large wildfires that follow misguided forest fire suppression. Widespread deforestation, especially in the tropics (the "lungs of the earth"), and coastal pollution that limits phytoplankton photosynthesis reduce the ability of the environment to remove CO<sub>2</sub> and clean the air. These various and pervasive activities are increasing CO<sub>2</sub> and methane levels, which are largely responsible for global climate change.

China recently became the global leader in emissions of CO<sub>2</sub>, and the United States is a close second. According to the Oak Ridge National Laboratory, of

North American releases of greenhouse emissions in 2002, approximately 92% were generated in the United States, and this was 26% of the world total (Marland *et al.*, 2005). A recent estimate indicates that half of the estimated 337 billion tons of CO<sub>2</sub> released globally due to human activities since 1751 has entered the environment since the mid-1970s (Boden *et al.*, 2010). The major source is fossil fuels, but cement production is becoming increasingly significant. During the 20th century, CO<sub>2</sub> emissions in the United States increased an estimated ninefold. According to the United Kingdom Department of Trade and Industry, China, which has a much higher human population and has been rapidly industrializing, has increased its CO<sub>2</sub> emissions in the same time frame by 6000-fold.

As ice caps melt due to the greater increases in temperature at the poles, more infrared is absorbed by the darker water and land, further aggravating warming. Additional methane is then released from lands previously covered with ice. Steady increases in average temperatures are also causing glacial retreat, and when the glaciers are finally depleted, downstream areas will dessicate. In addition, climate change has been linked to increasing extreme weather events (e.g., droughts, floods, and hurricanes) as well as to fires.

Rising global temperatures are also producing shifts in species distributions. Species, including arthropod vectors, that tolerate warmer temperatures are able to move farther from the equator and to higher elevations, commonly carrying diseases to previously unexposed and thus immunologically naïve wildlife. Climate change may also aggravate natural toxin problems, as noted later.

### DIRECT TOXICITY OF NUTRIENTS, STIMULATION OF HARMFUL ALGAL BLOOMS, AND FACILITATION OF LETHAL OUTBREAKS OF BOTULISM

Globally, the rate of transfer of atmospheric nitrogen (N) to the available nitrogen pool has doubled due to anthropogenic activities (Vitousek *et al.*, 1997). Nutrients, primarily N and phosphorus (P), enter environments from chemical fertilizers, manure, urine, sewage effluents, burning fossil fuels, fires, decay of plants and animals, and industrial processes such as pulp/paper milling and nitric acid production. Free nutrients are washed into water bodies and enter groundwater and aquifers, some of which feed streams, ponds, lakes, estuaries, bays, and oceans. Agricultural production of plants and animals is the primary source of excess nutrients in the environment. The lack of incentives and disincentives sufficient to substantially close the nutrient loop between

plants and animals is the heart of the problem. World consumption of fertilizers in 2005 and 2006 totaled 154 million tons. In 2003, the United States used 21 million tons of plant fertilizers, primarily as N, P, and potassium (K), which is approximately the same level as in 1976. Similarly, fertilizer use in other developed nations has plateaued, having reached a point where further inputs do not increase yields. However, as the human population and economic expansion increase, demand for animal protein, and thus production of animal feed, may prompt an expanded agricultural footprint with more extensive use of fertilizers, especially in developing countries.

Forms of inorganic N, including ammonia, ammonium, nitrate, and nitrite, are directly toxic to aquatic life, including invertebrates, fish, and amphibians. For example, ammonium nitrate fertilizer produced toxic effects in tadpoles of American toads (*Bufo americanus*), western chorus frogs (*Pseudacris triseriata*), northern leopard frogs (*Rana pipiens*), and green frogs (*Rana clamitans*) at concentrations found in the agricultural environment (Hecnar, 1995). Twenty percent of more than 8000 water samples collected from the Great Lakes watershed exceeded concentrations considered toxic to amphibians (Rouse *et al.*, 1999). High N concentrations contributed to lower amphibian reproductive success and reduced species richness in wetlands in agricultural areas (Knutson *et al.*, 2004).

Nutrient contamination can also set the stage for production of toxins by certain blue-green algae (cyanobacteria). Examples of cyanobacterial neurotoxins include anatoxin-A, anatoxin-A(s), saxitoxin, and neosaxitoxin. An as yet unidentified cyanobacterial neurotoxin is believed to account for avian vacuolar myelopathy (AVM). Evidence suggests that a cyanobacterium in the order Stigonematales produces a toxin as it grows on an exotic invasive macrophyte, *Hydrilla*, which is consumed by birds such as coots and ducks, resulting in AVM (Birrenkott *et al.*, 2004; Williams *et al.*, 2007). Eagles are subsequently exposed to the suspected toxin in poisoned prey.

In addition to producing neurotoxins, cyanobacteria may produce hepatotoxins, such as microcystins, nodularin, and cylindrospermopsin. Both neurotoxic and hepatotoxic cyanobacteria have been implicated in die-offs of lesser flamingos, an obligate algal feeder. However, additional research is needed before the importance of cyanobacterial toxins in long-term downward population trends of lesser flamingos can be deduced.

Although harmful marine algal blooms seem to be causing emerging toxicoses worldwide, and the causes are not adequately understood, the organisms require available nutrients. Among marine phycotoxin producers are diatoms that produce neurotoxic domoic acid and



different dinoflagellates that produce saxitoxins, ciguatera toxins, and brevetoxins. Brevetoxin exposures via aerosols and ingestion of sea grasses contaminated with the toxins seem to account for repeated major death losses in manatees along the southwest coast of Florida. Humans are also harmed by inhalation of brevetoxin-containing aerosols. Of great importance is that shellfish and finfish accumulate these various marine phycotoxins, and their ingestion leads to secondary poisoning in humans, cormorants, pinnipeds, and cetaceans (e.g., *Tursiops truncatus*) (Van Dolah, 2000; Flewelling *et al.*, 2005).

Free nutrients can also prompt growth of algae and cyanobacteria that smother and absorb essential UV needed by other photosynthetic species. Even nontoxic algal blooms can thereby cause massive alterations in ecosystems of lakes, estuaries, and coral reefs. Overgrowth of algae, cyanobacteria, and even macrophytes following nutrient pulses can result in decreased dissolved O<sub>2</sub> concentrations as a consequence of respiration of the photosynthetic organisms (e.g., at nighttime and during cloudy weather). Also, metabolism of dead algae, cyanobacteria, and macrophytes can greatly reduce dissolved oxygen concentrations and undermine nutrient storage.

According to the U.S. Geological Survey National Wildlife Health Center, more than 52,000 botulism-related avian deaths occurred on the Great Lakes during 2002–2006. Free nutrients made available in the tissues of dead animals and used by *Clostridium botulinum* to produce neurotoxins that are often conveyed by maggots to birds have long been incriminated in a vicious cycle causing major die-offs from botulism in waterfowl. A green alga and exotic species may be among additional factors that now contribute to initiation of avian botulism outbreaks. Increased water clarity and nutrient release due to inefficient feeding by invasive zebra mussels (*Dreissena polymorpha*) and quagga mussels (*Dreissena rostriformis bugensis*) have fueled dense growths of *Cladophora*, a filamentous green alga in the Great Lakes. When *Cladophora* ultimately dies, *C. botulinum* can thrive in the anaerobic mass (Byappanahalli and Whitman, 2009). The toxin can also accumulate in the mussels as well as in exotic round gobies (*Apollonia melanostoma* (*Neogobius melanostomus*)), and ingestion of the contaminated mussels or round gobies has been implicated in die-offs of common loons (*Gavia immer*) as well as in mergansers and other aquatic feeding birds.

Many phytoplankton and bacterial species have shorter generation times at warmer temperatures. Therefore, global climate change may lead to increased microbial diseases and harmful algal blooms. Other chapters of this book on nutrients, phycotoxins, and botulism should be consulted to learn more about the manifestations, diagnostic criteria, management, and prevention of the respective toxicoses.

## PETROLEUM DISASTERS, WILDLIFE TOXICOLOGY, AND HUMAN IMPACTS

Many of the effects of petroleum on wildlife are similar to those seen with domestic animals; therefore, readers who want to learn more about the impacts of petroleum on animal health should also examine the chapter on petroleum toxicity. Here, we provide examples of how petroleum can pose such extreme challenges to the health of wildlife and describe how some of its effects on wildlife are unique.

On March 24, 1989, the *Exxon Valdez* hit a reef, spewing almost 11 million gallons of crude oil into the largely pristine Prince William Sound in Alaska. Exxon Corporation (now Exxon Mobil), the ship's owner, the Alyeska Pipeline Service Company, a consortium of oil companies with interests in Alaska, as well as state and federal disaster response groups were criticized (and Exxon and Alyeska were eventually sued) as being wholly unprepared to provide a rapid and effective response for such a disaster as required by law. Confusion, poor communication, ineffective coordination, lack of sufficient personnel, inadequate equipment and supplies for a spill of that magnitude, and remoteness of the location hampered efforts to contain and begin effective cleanup for the first 2 days, and thereafter rough seas created further difficulties. The ecological and socioeconomic impacts were immense, and responses from conservation organizations and agencies, academic institutions, mass media, and the public were unprecedented. Frustrated by a lack of action on the part of industry and government, local citizens began to clean up what oil they could with resources at hand. Within hours of the spill, scientists arrived to begin documenting environmental impacts of the spill. Particularly sensitive environments were quickly identified and prioritized for protection and cleanup. Crews began to arrive from throughout the world to help clean oil-covered beaches, seabirds, and sea otters. Untold numbers of fish and wildlife died from direct impacts of oiling. An estimated 36,000 birds and 1000 sea otters were eventually treated for oil exposure. Approximately 25% of the Harlequin ducks in affected areas died after the spill, and the best estimate for time to recovery of the population was deemed to be 24 years (Iverson and Esler, 2010). Monson *et al.* (2000) detected reduced survival of sea otters in the 9 years following the incident, with some of the adverse effects noted in individuals born after the spill. According to the Exxon Valdez Oil Spill Trustee Council, the populations of only 4 of 11 bird species impacted were recovering or had recovered as of 2002. Spraying the oil with hot water caused penetration of sediments, prolonging effects. Impacts persist today (<http://www.evostc.state.ak.us/universal/documents/NEPA/Final%20SEIS%208-26.pdf>).

Populations of herring, a keystone species in the Prince William Sound's food chain and the crown jewel of the local commercial fishery, have not recovered. In addition to the economic losses to fisheries and tourism must be added nonmarket values (i.e., "replacement costs" of wildlife) as well as the costs of increases in cancers, respiratory illness, mental illness, and violence in the wake of the ecological and economic disaster. Although Exxon paid for some of the cleanup and lost earnings, the ecological and social impacts continue. In response to the *Exxon Valdez* disaster, the Oil Pollution Act of 1990 required increased spill preparedness and the phase-in of double-walled tanker ships, which greatly reduce chances of a spill when tankers run aground or hit an iceberg or reef. Unfortunately, the petroleum industry found loopholes in the law and effectively delayed the deadline for new or retrofitted tankers until 2015. Additional information on the history, current activities, and future plans surrounding this disaster is available at the *Exxon Valdez* Oil Spill Trustee Council's website (<http://www.evostc.state.ak.us>) and the U.S. EPA's *Exxon Valdez* Oil Spill Profile (<http://www.epa.gov/oilspill/exxon.htm>). Even now, surface and subsurface oil remain in some areas, poisoning aquatic life and slowing ecosystem recovery.

Although oil spills are preventable, they continue to occur, causing illnesses and mortality events in wildlife, which prompt extreme efforts by responders. Oil companies have sometimes paid out large sums to offset some of the impacts, but the costs to date have not triggered actions that reliably prevent spills from occurring.

Engineering flaws, poor preparedness for spill disasters, and delays and incoordination among responders were again evident during the British Petroleum Deepwater Horizon leak into the Gulf of Mexico in the spring and summer of 2010. In this case, areas of shoreline were partially protected by burning off oil, containment with booms, aspiration, physical removal on the land, and the offshore addition of dispersants of undefined composition in terms of specifics although they included solvents and detergents. Responders obtained and froze dead animals for necropsies, which continued 1 year after the spill. During and after the spill, rescue crews undertook triage including euthanasia of severely affected wildlife. Animals deemed to have reasonable prospects for survival were given diagnostic assessments, supportive care and time for acclimation and partial recovery, followed by extensive washing by well-trained crews and then more supportive care. Such efforts helped some of the affected wildlife, especially birds, to be returned to the wild. Although pelicans and other waterbirds were most often seen in media reports, among the more grave concerns were impacts to endangered sea turtles. Not only did the animals experience skin and respiratory irritation but also the dark-colored

oil coated their bodies and in the hot sun of the gulf area they became extremely hyperthermic. Some of the turtles were captured and treated, and death losses were somewhat curtailed. In addition, according to the National Oceanic and Atmospheric Administration, 274 sea turtle nests on the Gulf Coast were translocated to the east coast of Florida to avoid the impacts of oil pollution (<http://www.nmfs.noaa.gov/pr/health/oilspill/turtles.htm>).

Unfortunately, petroleum spills seem to receive far more attention from the media, corporations, and governments when they occur in developed countries compared to the developing world. A case in point is Nigeria, where, since the 1940s, the oil-rich Niger Delta has experienced oil spills that dwarf the 2010 Deepwater Horizon leak. With a corrupt government that has been unresponsive at best, oil companies that may be complicit in government violence against local peoples, native ethnic peoples receiving little if any compensation for oil extraction and related environmental degradation, associated unrest and protests that have turned violent, banning of public gatherings, and deaths including execution of leading activists for the Ogoni people of the Delta, the oil often seems to be more of a curse than a blessing to the Nigerian people. Currently, militant attacks on oil infrastructure have continued to aggravate spills, and the natural capital of the forests and waters continues to be replaced with pollution and oil infrastructure – leaving local people poor, sick, and discontented.

Waterbirds exposed to petroleum can lose the insulation and buoyancy of their feathers and, in cold water, can experience lethal hypothermia (Leighton, 1993). They can lose the ability to fly to feeding areas and sources of freshwater, resulting in starvation and dehydration. Petroleum toxicosis in birds can be associated with skin irritation, respiratory tract and ocular damage, neurologic dysfunction, and inhalation pneumonia. Birds with petroleum poisoning may also develop marked immunotoxicity with immunosuppression, and they often become progressively stressed and debilitated. They may experience digestive system irritation and even hemorrhagic gastroenteritis. They also may have renal failure, visceral gout, and hepatopathy with increased mixed function oxidase activities and associated alterations in the metabolism of endogenous and exogenous substrates – some of which result in further illness. Birds have repeatedly been found to develop hemolysis after exposure to petroleum. It has been suggested that certain polyaromatic hydrocarbons in a subset of petroleum sources may account for this effect. Consistent with oxidative injury, such birds may have elevated methemoglobin concentrations and Heinz body formation. Ultimately, such birds as well as fish may experience anemia and hemosiderosis (Khan and Nag,

1993). In fact, examinations of 560 common murres after the Apex Houston oil spill in February 1986 revealed packed cell volumes that were depressed by as much as 80%. As with other species, obtaining specimens of the oil from the animals and from the environment can be essential in ascribing liability.

Avian reproduction may be impacted by oil spills. Extremely small amounts of oil on eggshells can readily result in embryo mortality. Thus, oil transferred by minimally exposed parents to the eggs that they are incubating can be lethal to their offspring. Reproductive toxicity can also arise from exposure of adults to oil prior to egg laying because the eggs they lay may have reduced hatching and the surviving young may experience less than normal survival rates.

A current and growing concern pertains to placing oil fields in sensitive arctic and marine environments. A notable example is the Arctic Ocean off the north shore of Alaska. Oil spills in such areas may have immense adverse impacts on marine mammal populations and indigenous human communities.

It is clear that environmental and ecotoxicological problems related to petroleum including oil spills and global warming from CO<sub>2</sub> will continue for the foreseeable future. However, the problems do not stop there. Emissions during oil refining and polycyclic aromatics in the raw material and an array of products of combustion are also major concerns.

## AROMATIC HYDROCARBONS

Aromatic hydrocarbons have ring structures that confer the name to this group of toxicants. Included are both single ring and multiring (polyaromatic) structures. Petroleum includes a wide array of aliphatic as well as aromatic hydrocarbons. Aromatic hydrocarbons are also present at high concentrations in coal tar and are formed during incomplete combustion of coal, oil, gas, garbage, and other materials. Among the best known polycyclic aromatic hydrocarbons (PAHs) is benzo(*a*)pyrene, which is a component of cigarette smoke. Also of concern are volatile aromatic compounds such as benzene. Many of the aromatic hydrocarbons are oxidized by P450 enzymes in lung, liver, and other tissues to form reactive epoxide derivatives that form adducts to DNA, often resulting in mutations that have the potential to cause cancers as well as heritable defects. For example, both benzo(*a*)pyrene and benzene are known human carcinogens.

Another example of environmental contamination with aromatic hydrocarbons is that of the aluminum smelters of Quebec, Canada. Using the obsolete Soderberg method technologies, the smelting factories

released huge amounts of PAHs to the St. Lawrence estuary, where the compounds settled into sediments. Many species, including beluga whales, feed on benthic organisms. The PAHs, along with PCBs and other contaminants, were subsequently incriminated in digestive tract cancers of beluga whales as well as carcinogenesis in the local human population (Martineau *et al.*, 2002). After press coverage regarding the relationships among the contaminants and cancers, the aluminum industry of the area decommissioned the contaminating plants and installed cleaner technology ahead of their previously announced schedule.

The aryl hydrocarbon (Ah) receptor is a critical site of action of coplanar polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs) and PCBs. However, it should be kept in mind that the Ah receptor is activated not only by such compounds but also by unsubstituted aryl hydrocarbons. Accordingly, some of the impacts of the compounds discussed later are also catalyzed by PAHs.

## PERSISTENT ORGANIC POLLUTANTS

PCBs were marketed under the names Aroclor, Kanechlor, Clophen, and others throughout the world. The products consisted of mixtures containing more or less chlorine. PCBs came into widespread use in the 1930s and were banned in the 1970s. They were employed in electrical insulating, hydraulic and lubricating fluids, carbonless carbon paper, paints, and for many other uses. The array of structures of various PCB congeners in commercial mixtures influences their environmental and metabolic fate as well as their toxicity. Because of their widespread use, improper disposal, and semivolatile behavior, they have been released from equipment, soils, and sediments at contaminated locations and transported up to great distances in the atmosphere before condensation and deposition, often over cool surfaces such as large bodies of water. Thus, large lakes, seas, oceans, and glaciers have become repositories for such chemicals. This "global distillation" process largely accounts for the comparatively high concentration of persistent organochlorines in polar ocean food webs. Other semivolatile chemicals, such as organochlorine insecticides, the fungicide hexachlorobenzene, and PCDDs and PCDFs, which are toxic byproducts of organochlorine synthesis, manufacturing processes such as the Kraft paper bleaching process, and poorly controlled incineration, have similar environmental fates.

Most dioxin entering the environment today originates through incineration of products containing chlorine (Weber *et al.*, 2008). Today, Americans are exposed



primarily via consumption of beef and dairy products as a result of atmospheric deposition on foodstuffs. Fortunately, in developed nations such as the United States, efforts to limit emissions from municipal, medical, and commercial incinerators have substantially reduced atmospheric dioxin emissions.

PCBs, PCDDs, and PCDFs are uncharged molecules, and the more highly chlorinated congeners tend to be highly soluble in body lipids and difficult to metabolize and excrete. As a result, they are readily biomagnified in aquatic food webs, leaving top carnivores – especially in freshwater systems and contaminated harbors – at high risk of contamination and toxic impacts.

PCBs, PCDDs, and PCDFs can have serious effects on the immune, nervous, and reproductive systems, especially those of developing organisms. Such compounds can also reduce control of impulsive behaviors, impair learning, cause liver damage, and disrupt reproductive and thyroid hormone functions. For example, exposure of captive mink to PCBs in Great Lakes fish resulted in reproductive failure. Cleanup of “hot spots” of contamination and feeding the animals a much less contaminated diet enabled successful reproduction. Also, environmental exposures of Great Lakes fish-eating birds to organochlorines, including dioxins and dioxin-like compounds such as PCBs, resulted in embryo and chick mortality, growth retardation, and deformities associated with edema, hepatomegaly, gastroschisis, and other lesions – otherwise known as the Great Lakes embryo mortality, edema, and deformity syndrome, or GLEMEDS (Gilbertson *et al.*, 1991). Again, by curtailing emissions and cleaning up contaminated soils and sediments, this problem has been greatly reduced.

Whereas the concentrations of most organochlorine contaminants in the environment are decreasing, those of flame-retardant polybrominated diphenyl ethers (PBDEs) are increasing. These compounds are incorporated into foam cushions in furniture, fabrics, electronics cases, plastics, and other materials to reduce the risks of fires. Among the toxic effects of PBDEs are impairments in neurologic development, learning, attention, thyroid function, and spermatogenesis (Darnierud, 2003; Muirhead *et al.*, 2006). According to Environment Canada, concentrations of PBDEs in Great Lakes fish are showing marked and consistent increases ([http://www.on.ec.gc.ca/csl/fich/fich003\\_002\\_e.html](http://www.on.ec.gc.ca/csl/fich/fich003_002_e.html)). Also, killer whales (*Orcinus orca*) of the Pacific Northwest have body burdens well above those associated with toxic effects in other species (Ross, 2006). Although some of the most toxic congeners of PBDEs have been banned in areas of Europe and voluntarily withdrawn in the United States, environmental contamination continues on an upward trend due to uncontrolled disposal of highly contaminated products.

## CONTAMINANTS FROM E-WASTES

Since the advent of the personal computer and throw-away televisions and stereos, e-wastes (i.e., discarded electronics) have been an ever-growing environmental issue – one that transcends political boundaries and has great impact on the poor in developing nations. According to the U.S. EPA, in 2007, the United States generated 2.25 million short tons of e-waste, which represented 2% of municipal waste streams. Of this, 82% by weight of televisions and computer components went to landfills, and much of the remainder was shipped to developing nations, especially China, India, and Ghana, where men, women, and children dismantle and mine them for the metals in the circuit boards, wiring, and other components.

At uncontrolled e-waste recycling sites, mountains of discarded and burning components leach chemicals into the air, soil, and water, where they find their way into biota. PCBs and PBDEs have been detected in the serum of a large portion of samples from multiple species of birds and especially herons in an e-waste recycling region of China (Luo *et al.*, 2009). The city of Guiyu, China, has become a poster child for e-waste problems, with reportedly the largest volume of e-waste on Earth. Recovery of metals over open flames without personal protection or environmental safeguards exposes workers, the communities, and the nearby environments to cadmium, lead, nickel, and other metals. Toxic levels of heavy metals have been found in the city's children (Zheng *et al.*, 2008). Burning of circuit boards and other plastic components releases plasticizers including bisphenol A, triphenyl phosphate, palmitic and stearic acids, brominated flame retardants, and the highly chlorinated flame retardant Dechlorane Plus (Dechlorane A) into the air, soils, and tissues of resident people and animals near the recycling facilities, potentially causing oxidative stress, reproductive and developmental problems, and altered thyroid function.

In addition to causing human poisoning and environmental pollution, the current methods of e-waste recycling in developing countries squander both energy and other materials. Efforts are under way to stem such practices by increasing the recovery, recycling, repurposing, and refurbishment of more items in countries of origin and sale, as well as providing developing nations and communities with means to recover materials in a safe and efficient manner. Ultimately, designing products so that, when they lose their usefulness, their materials are readily reconverted into useful new materials (i.e., cradle-to-cradle technology) should resolve major e-waste-related and many other environmental contamination problems (McDonough and Braungart, 2002).



## PESTICIDES

World pesticide usage was approximately 5.1 billion pounds in 2005 and 2006. The United States alone used approximately 25% of the total herbicides, as well as 10% of the insecticides, 14% of the fungicides, and 26% of the other types of pesticides ([http://www.epa.gov/opp00001/pestsales/07pestsales/market\\_estimates2007.pdf](http://www.epa.gov/opp00001/pestsales/07pestsales/market_estimates2007.pdf)). Agriculture accounts for the largest proportion of total conventional pesticides applied (70–80%) in the United States, and the most highly used compounds include glyphosate, atrazine, metam sodium, metolachlor-S, acetachlor, dichloropropene, 2,4-D, methyl bromide, and chloropicrine. Many pesticides, including some no longer in use, have important ecotoxicological impacts. Given the numbers of pesticide products used, in this chapter, we can at best highlight some instructive examples. Other chapters in this book, as well as books devoted entirely to pesticide toxicology and ecotoxicology, the primary research literature, and government reports, should also be consulted.

### Organochlorine insecticides

As noted in part previously, the widespread use of organochlorine insecticides (OCIs), DDT, aldrin, chlordane, dieldrin, heptachlor, and others began after World War II. These compounds were initially considered a boon to agriculture and public health because they were both effective and persistent. However, because of their persistence, pests soon began to develop genetic resistance. The persistent OCIs also poisoned insect predators so that the pests had greater impacts than before. These phenomena triggered higher application rates and, eventually, the continual development of new compounds. As with other toxicants, the OCIs have different effects via actions at a range of types of molecular receptors in the body.

The insecticidal properties of OCIs relate mainly to neurotoxicity. The molecular receptors are highly conserved and thus both invertebrates and vertebrates are susceptible. DDT increases sodium conductance across nerve cell membranes, increasing excitability and resulting in tremors and the potential for seizures. The cyclodiene pesticides such as aldrin, chlordane, dieldrin, endrin, and heptachlor, the structures of which differ markedly from that of DDT, exert their influence most prominently at  $\gamma$ -aminobutyric acid (GABA) receptors in the brain, thereby reducing chloride conductance. In general, the cyclodienes are more acutely toxic than DDT. Consequently, cyclodienes such as aldrin and dieldrin have resulted in direct mortality in predatory birds,

such as sparrowhawks and kestrels (Newton *et al.*, 1992). This mortality, when combined with sublethal effects such as starvation and accidents due to neurological deficits and reduced productivity due to eggshell thinning caused by DDE exposure, soon led to population declines in these birds (Walker and Newton, 1998).

DDE often co-occurs in avian eggs with PCBs, and both are embryotoxic endocrine disruptors. Together, they may interact to contribute to decreased productivity in populations of waterbirds such as bald eagles (Bowerman *et al.*, 2003).

As with PCBs and dioxins, the slow biodegradation rate and high lipid solubility of many OCIs lead to biomagnification in food webs. Predatory animals thus often accumulate very high concentrations, and the nursing young of top predators feeding in contaminated aquatic ecosystems can be massively exposed. Among the species most at risk are marine mammals, the young of which may consume milk with extremely high fat content (e.g., up to 60% fat).

Fortunately, after decades since most of the compounds were banned, restricted, or voluntarily withdrawn, environmental concentrations of OCIs in most of the world have greatly declined. As a result, impacts of OCIs on wildlife have significantly, but not totally, abated.

### Concurrent exposures of frogs to DDT analogs and other persistent organochlorines

Reeder *et al.* (2005) suggested that populations of frogs may have been negatively impacted by DDT and PCBs. They examined spatial and temporal patterns of intersexuality in cricket frogs collected in Illinois from 1852 to 2001. In contrast to the preorganochlorine era (1852–1929), the percentage of intersex cricket frogs markedly increased during a time of industrialization and initial uses of PCBs (1930–1945), was highest during the peak of manufacture and use of DDT and PCBs (1946–1959), began declining with increased public concern and environmental regulation that led to restrictions and the eventual ban on the use of DDT in the United States (1960–1979), and continued to decline through the period of gradual reductions in environmental residues of OCIs and PCBs in the midwestern United States (1980–2001).

### Cholinesterase-inhibiting insecticides

Organophosphorous (OP) and carbamate insecticides such as diazinon, chlorpyrifos, malathion, and carbofuran are cholinesterase-inhibiting compounds. As such, they prevent breakdown of acetylcholine after transmission of a nerve impulse. The first OP cholinesterase

inhibitors were developed as chemical weapons during World War II. After the war, as pests developed resistance to OCIs and their use was restricted, the use of OP compounds increased. The OP compounds are not persistent in the environment compared to most OCIs, but they are broad-spectrum insecticides, and many exert potent acute toxicity. Human deaths from mishandling occur annually. Secondary poisonings can occur when predators consume invertebrates or vertebrate wildlife that were poisoned by OP and, less often, carbamate insecticides. In one of the best documented secondary poisoning events, approximately 6000 wintering Swainson's hawks (*Buteo swainsoni*) were poisoned in Argentina during 1995 and 1996 after they fed on grasshoppers that had been sprayed with the OP insecticide monocrotophos (Goldstein *et al.*, 1999). In another incident, hundreds of laughing gulls (*Larus atricilla*) were poisoned after consuming insects that were killed or debilitated by application of parathion to cotton fields (White *et al.*, 1979). The mortality included nestlings that died after being fed contaminated prey by adult gulls. It is likely that large numbers of individual poisonings of birds and other species by OP insecticides go unnoticed following the intended use of licensed products. Organophosphorous insecticides have also been used illegally as avicides to protect grain crops from bird depredation and as canicides to kill coyotes (*Canis latrans*) (White *et al.*, 1989; Wobeser *et al.*, 2004). Such misuse has resulted in additional mortalities in predatory birds and mammals that fed on the carcasses of the illegally poisoned species.

Two OP insecticides that were previously used very widely, diazinon and chlorpyrifos, are now severely restricted in the United States. Diazinon, which had been widely used on golf courses and lawns, was infamous for its lethality to birds. Chlorpyrifos had been routinely applied to homes, for termite and pest control, and in crop agricultural applications. The removal of diazinon and chlorpyrifos from household products was followed by almost immediate reductions in concentrations in umbilical cord blood and increased birth weights of babies of low-income families. As reviewed by Barrett (2007), the basis for withdrawal of the registration of chlorpyrifos from household use related to findings of lifelong neurotoxicity in laboratory animals exposed during development. Later, effects that were deemed consistent with the predictions from that research were noted in children. Whether such effects also occur in wildlife exposed under field conditions warrants concern.

Granular carbofuran pellets have been consumed by birds that apparently mistook them for seeds, and one pellet of this carbamate insecticide is enough to kill a bird (Balcomb *et al.*, 1984). In recent decades, carbofuran was the insecticide most commonly associated with

wildlife pesticide poisoning events (e.g., red-winged blackbirds (*Agelaius phoeniceus*), bald eagles (*Haliaeetus leucocephalus*), golden eagles (*Aquila chrysaetos*), coyotes, red foxes (*Vulpes vulpes*), red-tailed hawks (*Buteo jamaicensis*), buzzards (*Buteo buteo*), and herons), and it was implicated in declines of burrowing owls (*Athene cunicularia*) (Hunt *et al.*, 1995; Blus, 1996; Elliot *et al.*, 1996; Wobeser *et al.*, 2004). Fortunately for wildlife in the United States, a phaseout of the granular formulation of carbofuran began in 1991, and registrations for most solid forms were canceled. Ongoing concerns with regard to carbofuran include its availability in other countries and its misuse in malicious poisonings to kill wildlife in African nations.

### Other insecticides

Pyrethrins from chrysanthemums and structurally related synthetic insecticides (pyrethroids) bind to sodium channels, preventing closure, and/or to GABA-mediated chloride channels inhibiting chloride influx, resulting in excessive depolarizations culminating in tremors or seizures. These chemicals are generally of low toxicity to mammals and birds; however, they exert potent acute toxicity to arthropods, including non-target taxa such as spiders, parasitoids, and bees. Although early formulations had a short half-life, measured in hours in direct sunlight, newer compounds are designed to be more photostable, extending their effectiveness for up to 10 days outdoors, which increases environmental risks associated with their use. Synthetic pyrethroids often exhibit high acute toxicity to tadpoles and fish. For example, several pyrethroids have LC<sub>50</sub> values for fish of less than 1 µg/L in acute studies, and they can cause chronic toxicity at concentrations as low as 0.01 or even 0.001 µg/L (Bradbury and Coats, 1989).

A variety of other types of products are used for control of insects, arachnids (e.g., mites and ticks), and other "pests." Some are nerve poisons, and others inhibit energy production, chitin synthesis, metamorphosis, water balance, or growth. A number of lower risk pesticides include microbes (e.g., the soil bacterium *Bacillus thuringiensis* (*Bt*) for control of moths and mosquitoes), microbial products (e.g., spinosyn, which is a fermentation product of a soil actinomycete for control of caterpillars), a range of other "natural chemicals" (e.g., pheromones, florals, and sulfur), and "plant-incorporated protectants" (e.g., transgenic crops that may produce toxins originally synthesized in bacteria such as *Bt*). Many of these newer methods of pest control are more environmentally benign alternatives to traditional insecticides. Nevertheless, because they disrupt normal physiological processes of both target and

non-target species, each carries potential environmental risks that are only partially understood.

### Indirect effects of insecticides

In addition to direct toxic effects to avian wildlife, insecticides may reduce populations of non-target invertebrates used as food by birds, which may require increased foraging effort or prey switching by adults feeding their offspring (Morris *et al.*, 2005). Additional research in this topic area is needed.

Exposures to insecticides might also influence trematode infections in frogs by impacting populations of benthic and planktonic predators of motile stages of the parasites, including cercariae. Invertebrates, including hydra, copepods, daphnids, and especially damselfly larvae and dragonfly larvae, attacked, consumed, and digested trematode cercariae in a controlled setting (Schotthoefer *et al.*, 2007). Insecticide-mediated changes in invertebrate community structure might therefore release cercariae or other trematode life stages from this predation pressure. The overall importance of these “micropredators” as determinants of infective loads of cercariae in natural and human-altered aquatic environments, and their susceptibility relative to the parasites themselves and the other hosts involved in their life cycles, remains to be thoroughly investigated. Additional information on trematodes, amphibians, and toxicant interactions is presented later.

### Herbicides

During 2000, herbicides represented 36% of the world pesticide market, and in 2001, the agricultural sector accounted for 78% of herbicide use in the United States. Herbicides are often considered benign with regard to impacts on animals; however, many such compounds can reach concentrations in the environment that lead to direct toxicoses, and they can secondarily trigger other lethal mechanisms.

Atrazine is perhaps the world's most used agricultural herbicide. According to the U.S. EPA, an estimated 74–80 million pounds were applied in the United States alone in 2001. Atrazine and its degradation products have been found in air, rainwater, surface water, and groundwater.

Exposure of African clawed frogs (*Xenopus laevis*) to atrazine at 320 µg/L resulted in increased mortality. In different amphibian species exposed to a range of test concentrations found in the environment, atrazine has been associated with reduced survival of tadpoles, increased length of larval period, and reduced size at metamorphosis (Diana *et al.*, 2000; Sullivan and Spence, 2003; Storrs and Kiesecker, 2004). Atrazine and other

herbicides may exert such effects in part by reducing dissolved oxygen and algal food needed by tadpoles. Reduced size at metamorphosis is likely to be followed by reduced survival and impaired reproductive fitness.

The registration permitting the sale of atrazine was not renewed by several European Union countries. Among concerns regarding this herbicide is endocrine disruption. Reeder *et al.* (1998) associated detection of atrazine with intersex gonads in cricket frogs (*Acris crepitans*) in Illinois. Other workers subsequently found that low concentrations of atrazine affected gonadal development, produced hermaphroditism, and reduced the laryngeal muscle in males (needed for calling to attract females and to ward off competing males). At a range of test concentrations that can be found in the environment, atrazine was associated with gonadal dysgenesis, reduced spermatogenesis, impaired reproductive behaviors, and sex reversal (Tavera-Mendoza *et al.*, 2001; Hayes *et al.*, 2010). Although other workers have suggested that intersex is common in unexposed frogs, in some such reports, the animals in control groups or at reference sites were also exposed to low concentrations of atrazine (Coady *et al.*, 2004; Smith *et al.*, 2005).

Declines in amphibians in the wild have impacts on other species. Reducing tadpoles prevents them from consuming algal food, which leaves excess algae in waters, causing turbidity that can impact on a host of other species. Also, in some forest ecosystems, amphibian biomass in late summer can exceed that of all other vertebrate groups combined. In these and other types of ecosystems, tadpoles and frogs serve as a vital source of food for other amphibians, reptiles, birds, and mammals. Without the amphibians as a food source, other species can experience malnutrition and precipitous declines.

Glyphosate affects a broad spectrum of plants and has become the most used herbicide in the United States. Glyphosate has minimal toxicity to mammals, but surfactants added to some formulations to promote adherence to plant surfaces greatly increase product toxicity (Goldstein *et al.*, 2005). Thus, some formulations (those not intended for aquatic vegetation control) can result in direct mortality of larval and juvenile amphibians and subsequent loss of biodiversity. Glyphosate readily adsorbs to soil, is rapidly degraded by bacteria, and has limited bioavailability. Nevertheless, due to its high water solubility, runoff can result in detectable concentrations in surface waters. Of course, glyphosate is sold in formulations intended to control not only terrestrial but also aquatic vegetation. These and other herbicides kill both target and non-target plants, which alters ecosystem functioning. Also of concern is that a range of stressors may enhance the toxicity of pesticides, and predatory stress substantially increased the lethality of Roundup (a popular glyphosate product) to larval amphibians (Relyea, 2005).



## Interactions among pesticides, nutrients, and pathogens

Studies examining mixtures of pesticides have found additive, synergistic, or antagonistic effects on organisms, depending on the chemicals used, their concentrations, the test organisms, and the laboratory conditions. Nutrients may also interact with pesticides to produce interactive toxicity. Also, pesticides and nutrients can influence the incidence and intensity of infectious diseases in wild animals.

Nutrients and pesticides, coupled with habitat change, may help explain some of the recent outbreaks of supernumerary limbs and declines in frogs. Waterbirds are the definitive hosts of trematodes that rely on amphibians as intermediate hosts. In the life cycles of these parasites, sexual reproduction occurs in the birds and eggs are shed in their excreta. The first intermediate hosts are snails, in which massive asexual reproduction occurs. The second intermediate hosts are tadpoles, and when the amphibians are consumed by birds, the life cycle is completed. Because of ubiquitous drainage of aquatic habitats, waterbirds often crowd into remnant water bodies, providing potentially higher inputs of infective ova. Excess nutrient concentrations can increase algae and periphyton that comprise the main food of snails. Johnson *et al.* (2002) found positive relationships among nutrient pollution, snail populations, the frequency of limb malformations, and the intensity of infections of post-metamorphic frogs with *Ribeiroia* trematodes. While *Ribeiroia* infections may produce limb deformities, trematodes in the genus *Echinostoma* may cause potentially debilitating kidney infections in frogs (Fried *et al.*, 1997; Schotthoefer *et al.*, 2003a; Beasley *et al.*, 2005). Infections by either of these trematode groups in early stage tadpoles can cause nearly 100% death losses (Schotthoefer *et al.*, 2003a,b). Moreover, sublethal trematode infections may also make tadpoles less tolerant of other stressors.

Studies by our group suggested complex interactions among herbicides, nutrients, and trematode infections of amphibians in agricultural regions (Beasley *et al.*, 2005). In herbicide-impacted farm ponds, recruitment of juvenile cricket frogs was reduced, and trematode infections in the surviving frogs were greatly increased. Sousa and Grosholz (1991) suggested that more complex habitat structure impedes parasite transmission, and Beasley *et al.* (2005) hypothesized that severe trematode infections in frogs were due to interacting factors related to fewer plants in the water. In such simplified ecosystems, predation on amphibians by birds, reptiles, and fish would likely be facilitated, and motile cercariae might more readily find the surviving tadpoles so that they could receive a high infective load of trematodes. In a field study that examined more than 240 variables, atrazine and phosphate contamination was by far most closely

correlated with infections of leopard frogs (*R. pipiens*) with trematode infections. Also, studies of microcosms showed that atrazine removed phytoplankton, which increased the clarity of water; reduced macrophytes; increased periphyton and snails; immunosuppressed tadpoles; and increased their infection intensities with trematodes (Rohr *et al.*, 2008). Other research groups have also linked pesticide exposure to immunosuppression and increased susceptibility of amphibians to trematode infections (Kiesecker, 2002; Linzey *et al.*, 2003).

## Anticoagulant rodenticides

Anticoagulant rodenticides such as brodifacoum and bromadiolone inhibit blood clotting by inhibition of vitamin K epoxide reductase, an enzyme essential to the reuse of the vitamin in producing active forms of clotting proteins. Many such compounds are highly and acutely toxic. These rodenticides may directly impact non-target small mammal populations (Brakes and Smith, 2005). Also, secondary poisonings (relay toxicoses) have had impacts on birds of prey, such as buzzards, barn owls (*Tyto alba*), and red kites (*Milvus milvus*), as well as carnivorous mammals, such as mink (*Mustela vison*), polecats (*Mustela putorius*), and red foxes (Fournier-Chambrillon *et al.*, 2004; Ntampakis and Carter, 2005).

## Organotin biocides

Organotin biocides are widely used to coat pilings and ships to prevent the growth of barnacles, algae, and other organisms that deteriorate wood and/or create drag. Members of this environmentally persistent group of biocides are known for potent neurotoxicity as well as the capacity to induce “imposex” in mollusks, which may be manifested by sexual abnormalities and infertility in females (Ruiz *et al.*, 1998).

## ENDOCRINE-DISRUPTING MIXTURES IN SURFACE WATERS

Water bodies contaminated by persistent organic pollutants, detergents, plasticizers, pesticides, mercury, nitrate, other chemicals, sewage effluents, runoff from animal production systems, and ubiquitous cyanobacteria may induce apparent endocrine disruption in aquatic animals. Xenobiotics that influence hormone receptors include agents that (1) mimic effects of hormones at receptors (agonists), (2) interfere with normal hormonal activity at receptors (antagonists), (3) interfere with



synthesis of natural hormones, (4) accelerate metabolism or elimination of natural hormones, and/or (5) alter downstream biochemical processes that would otherwise follow normal receptor activity. Some agents can act through several of the previously mentioned mechanisms at different sites in the body. Also, agents that are weak agonists may have a net effect of receptor stimulation in immature animals and yet cause competitive inhibition of natural hormone activity in adults. In general, the developing young are most susceptible to the effects of endocrine disruptors, and effects on them often cause lifelong dysfunction.

A variety of endocrine-disrupting compounds have been detected in sewage effluent, including steroidal hormones, pesticides, breakdown products of surfactants and plasticizers, pharmaceuticals, and more (e.g., PCBs and dioxins). Kolpin *et al.* (2002) detected 82 compounds, 34 of which are known or suspected to have estrogenic activity, in 139 U.S. streams. In that study, 75% of the streams sampled had more than one of the compounds at detectable concentrations.

To our knowledge, the first study to establish a link between endocrine disruption and exposure to both sewage effluent and synthetic estrogens was that of Purdom *et al.* (1994). Following reports from fishermen of hermaphroditic fish in sewage lagoons, caged rainbow trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) were placed in sewage effluent in the field, and others were exposed to an oral contraceptive formulation in the laboratory. Marked increases in plasma vitellogenin (VTG) concentrations were observed, particularly in the trout. VTG is an egg-yolk precursor protein produced in the liver under control of estrogen. In adult fish, VTG is normally present in measurable amounts only in the blood or tissues of mature females. Thus, plasma VTG concentrations in males have been widely used as a marker of exposure to environmental estrogens. Induction of VTG, increased incidence of intersexuality (the presence of ova in testicular tissue), and reduced gamete production and quality have been observed in male fish collected below sewage outfalls. VTG responses are dose and time dependent: fish chronically exposed to low concentrations of effluent in river water had a lower threshold for VTG induction than did fish exposed for a shorter time period. Fish that are affected may show a sustained VTG response to estrogen exposure because plasma VTG concentrations in male fathead minnows remained elevated 21 days after exposure to estradiol (Panter *et al.*, 2000). Young male roach (*Rutilus rutilus*) exposed to graded dilutions of sewage effluent experienced VTG induction and feminization of reproductive ducts but no effects on germ cell development (Rodgers-Gray *et al.*, 2001). High levels of plasma VTG in male fish may be accompanied by kidney and liver damage. Increased liver mass, reduced gonad mass relative to body mass, testicular

damage, reduced gonopodium length (the modified male anal fin that is critical for sperm transfer in some species), and reduced serum testosterone concentrations have been observed in fish collected downstream of wastewater treatment plants (Panter *et al.*, 1998).

Natural estrogen (e.g., 17- $\beta$  estradiol) and ethinylestradiol (from birth control medications) are among the most commonly occurring and potent endocrine-disrupting compounds in effluents from treatment plants that receive domestic wastewater. However, sensitivity to estrogen exposure is species specific, highlighting the need for proper selection of species for monitoring purposes in regard to impacts on local fish communities (Purdum *et al.*, 1994; Thompson *et al.*, 2000). Increased VTG production has also been documented in mussels exposed to sewage effluent (Quinn *et al.*, 2004).

Research studies have documented a variety of endocrine-disrupting effects of steroidal hormones on fish, including reductions in (1) gonadosomatic indices (organ weights as a proportion of body weight), (2) the proportion of males with milt (seminal fluid containing sperm) and spawning tubercles, (3) gonopodium length, (4) courtship displays, (5) chasing/following behaviors, (6) copulation attempts, and (7) aggressiveness toward other males, and (8) fecundity (number of eggs) but not fertility (Doyle and Lim, 2002). Such effects would likely cause marked reductions in reproductive success.

Contaminants from livestock production are additional sources of environmental contamination with active hormones. Estradiol in ponds receiving water from cattle pastures was associated with increased VTG concentrations in female painted turtles (*Chrysemys picta*) (Irwin *et al.*, 2001). Also, cattle feedlots in Nebraska were found to cause endocrine disruption in both male and female fathead minnows (*Pimephales promelas*) in waters receiving runoff (Orlando *et al.*, 2004). In that study, males had reduced testicular size and testosterone synthesis, and they failed to develop normal secondary sex characteristics. Also, gonads of females had reduced *in vitro* estrogen:androgen ratios. Because of the complex mixtures of natural and synthetic hormones and their metabolites in the animal wastes, it was unclear what agents accounted for the masculinization of the fish, but the net changes were consistent with environmentally persistent and bioactive metabolites of the androgenic anabolic steroid trenbolone acetate, which is used widely in feedlot cattle.

Blooms of *Microcystis aeruginosa*, a common cyanobacterium in nutrient-enriched waters, could add yet another source of estrogenicity to water, as indicated by gene expression in exposed larval zebrafish (*Danio rerio*) (Rogers *et al.*, 2011). The estrogenic effects were not attributable to the often-encountered microcystin-LR contained in the cyanobacteria.

The long-term impacts of exposure to complex mixtures of endocrine-disrupting compounds on

populations of fish, frogs, and other aquatic life are not well understood. Grist *et al.* (2003) modeled the effects of ethynylestradiol exposure on populations of fathead minnows (*P. promelas*) and predicted that reduced fertility could impair population growth. Whether males with subtle intersex characters breed successfully in the wild and whether a population with fewer mature males can be sustained long term are areas of concern. With urbanization and a burgeoning human population, effluent-dominated streams (i.e., streams that contain mainly effluent from sewage treatment facilities) are becoming increasingly common. Accordingly, impacts of hormones and other substances that survive sewage treatment are a growing concern. Considering all of the endocrine disruptors as well as the many other pharmaceuticals, personal care products, and cleaning compounds that are resistant to biodegradation and thus pass through sewage treatment plants as parent compounds or active metabolites, there is a need for development and application of new technologies (Carballa *et al.*, 2004). The goal should be to ensure detoxification or physical removal of endocrine disruptors and other toxicants from sewage effluents. Similar attention is needed for animal production facilities.

## METALS, MINERALS, AND MINING

Other chapters of this book should also be examined to best gauge the impacts of metals, minerals, and mining on wildlife and ecosystems. Metals are electropositive elements that readily lose electrons to form cations. Some heavy metals, such as cadmium, lead, and mercury, have high atomic weights, have no known physiological role, and can produce toxic effects at low concentrations. Others, such as copper and zinc, are essential for life but are toxic at elevated concentrations. Metalloids are elements with properties intermediate between metals and nonmetals. Metalloids such as arsenic and nonmetals such as selenium can be toxic at relatively low doses. Of course, regular intake of low levels of selenium is essential for survival and good health.

Metals are usually complexed with other elements in the environment, and the form, or species, of a metal as well as its valence state are important determinants of toxicity. Trivalent chromium occurs naturally, and small amounts are thought to be necessary for optimal health. In contrast, hexavalent chromium is produced and used in industry and is highly toxic.

Irrigation, fertilizers, and pesticides can increase concentrations of toxic elements and salts in surface soils. Early pesticides included metal salts such as lead arsenate, and in some areas soils remain contaminated

from early use of these compounds. Also, mining and smelting, as well as processing and burning of pressure-treated wood have produced toxic environmental concentrations of metals and metalloids.

Because of persistence of mercury in the biosphere and because human and wildlife populations are already experiencing its toxic impacts, continued environmental loading, atmospheric transport, and deposition of this element are grave concerns. Mercury is a volatile metal that is released naturally into the biosphere from volcanic eruptions and exposed bedrock. Humans increase mercury exposures through ongoing use of obsolete technologies, in chloralkali plants that produce chlorine (being phased out in much of the world), coal burning, waste incineration, mining, smelting, and metal processing, as well as from reservoir development, dental wastes, and disposal of thermometers, barometers, electrical switches, and fluorescent lights.

In terms of wildlife impacts and long-term contamination of fisheries, an especially important source of mercury contamination is use of the liquid metal to capture gold from streambed deposits in artisanal gold production. Veiga and Baker (2004) reviewed human and environmental impacts of the artisanal uses of mercury for gold capture and estimated that approximately 1 or 2 g of mercury is lost for each gram of gold recovered. Poverty is a major driver of artisanal mining of gold, and poor and corrupt governments often fail to improve the plight of artisanal miners, their villages, and their regional environments. Among other locales, this is important in areas of South America, Southeast Asia, Indonesia, and Africa. Gold particles are brought up from the river sediments or dug from ore-laden soils that are washed and/or screened and added to liquid mercury to form an amalgam, but some mercury is lost during this process. The amalgam is then placed into a retort, or distillation vessel, that is heated to evaporate the mercury, leaving behind the gold. Often, these are open retorts, and little, if any, of the mercury is captured. The use of mercury to recover gold has resulted in the pollution of tributaries of large rivers such as the Amazon, Nile, Mekong, and Zambezi. Thus, mercury pollutes international waters that provide many people with water needed for drinking, bathing, washing, and food fish. Although the miners and their families may be exposed to dangerous levels of mercury during the recovery process, fish is the primary source of mercury exposure for human populations and wildlife along the rivers. Miners move on as gold becomes depleted, and the problem becomes more widespread. A great deal more needs to be done to prevent further mercury pollution and toxic impacts as a consequence of artisanal gold mining, including finding new sources of income for affected communities.

When mercuric salts and metallic mercury are metabolized by organisms in anaerobic sediments, they form

the more toxic methylmercury. The rate of conversion of other forms of mercury to methylmercury is much faster in certain warm climates that have favorable geochemistry (e.g., the Amazon basin and Florida) than in many areas more distant from the equator. Methylmercury bioaccumulates via the aquatic food chain, readily crosses the placenta and blood-brain barrier, and is highly toxic to the developing nervous system.

Methylmercury toxicoses in food chains of the Everglades were linked with high feather mercury concentrations, immunosuppression, same-sex pairing, and population declines in fish-eating birds (Sepulveda *et al.*, 1999; Spalding *et al.*, 2000; Adams and Frederick, 2008). Fortunately, removal of mercury from batteries and other sources entering a regional incinerator, that were the main source of mercury loading of the environment, resulted in rapid reductions in mercury contamination of fish and fish-eating birds, and this was followed by apparent improvement in their health and reproduction.

Cadmium is a toxic element used in Ni-Cd batteries and in certain pigments, plastic stabilizers, coatings, alloys, and electronics. It is also an impurity found in conjunction with and released during the processing of other metals such as copper, iron, and zinc. Cadmium also enters the environment from the manufacturing of steel, cement, and fertilizers, as well as the combustion of fossil fuels. The metal is readily taken up by some plants (e.g., leafy vegetables and rice) and is then ingested by humans and other animals in their food. Among the principal target organs of cadmium are bone and kidneys. Larison *et al.* (2000) monitored trace metals in the food web and tissues of white-tailed ptarmigan (*Lagopus leucurus*) in Colorado, and they concluded that trace amounts of cadmium can both undermine the health of individuals and alter the demographics and distributions of species.

Lead has been used in shotgun pellets, bullets, fishing sinkers, paint, batteries, gasoline, solder, water pipes, and other products. In the United States, one of the largest sources of lead in the atmosphere was alkylated lead added to gasoline to reduce engine preignition or “knocking.” To limit harmful emissions, beginning in 1975, passenger cars and light trucks manufactured in the United States included catalytic converters that required lead-free gas. The U.S. EPA therefore mandated a phase-down of lead concentrations from 2–3 to 1/10th g/gallon during 1977–1986. Prohibitions on lead in paint, water pipes, and tin cans were also enacted during this period. Leaded gasoline was still available for use in passenger automobiles in some areas of the United States until 1996, and it is still used in aviation, motorboat, farming, and racing vehicle gasoline. In the United States, reductions in lead use were reflected in a 37% decrease in mean blood lead concentrations of humans during 1976–1980 and a 41% reduction between

the periods 1988–1994 and 1999–2002 (Muntner *et al.*, 2005). However, lead exposures among children of some socioeconomic groups in the United States remain high due in part to contaminated dust, lead paint chips, lead dissolved from plumbing, and other sources. Worldwide, internal combustion engines are still a major source of environmental lead, although an increasing number of nations have banned or are beginning to restrict its usage in gasoline. Although lead shot to hunt waterfowl is now illegal in the United States and this has substantially reduced poisoning of waterfowl, lead used to hunt upland game has been a major cause of poisoning of scavengers, including highly endangered California condors. After the 22 remaining birds were brought into captivity and their offspring were eventually reintroduced to the wild, the birds were often poisoned by lead, leading to routine periodic capture and chelation, usually with calcium disodium EDTA (Church *et al.*, 2006). Ultimately, this resulted in regulatory and voluntary limits on the use of lead ammunition to hunt larger game in the range of California condors. Of course, scavengers and carnivores that feed on smaller species not protected by those measures, and scavengers and carnivores in other areas where lead ammunition continues to be used for all sizes of upland game, are still being poisoned by lead in carcasses and gut piles of game animals (Kelly and Johnson, 2011). Another important source of lead that affects wildlife is its continued use in fishing tackle, which poisons endangered loons, as well as pelicans, cormorants, and herons (Franson *et al.*, 2003). Concerned animal health professionals and others are urging a ban on lead in ammunition used in hunting as well as in fishing tackle.

Mining of metals and minerals often carries large environmental impacts. Not only do these activities leave the land degraded and scarred but also processes to remove the product of interest often result in the release of toxic elements or other chemicals into the environment. For example, sulfides released from newly exposed rock may combine with water and oxygen to form deadly sulfuric acid, which often makes its way into soils, groundwater, and streams. This acid has the added effect of leaching or dissolution of toxic elements such as arsenic, cadmium, lead, iron, and mercury from mine tailings or “waste” rock. The mining boom of the mid- to late 1800s in the American west left many miles of streams and rivers that are still contaminated with metals and sulfuric acid.

A long history of coal mining has also left a legacy of ecological degradation and toxicity. Concerns regarding the environmental and aesthetic effects of surface coal mining led to the Surface Mining Control and Reclamation Act of 1977. This law required mine owners to reduce impacts of surface mining by “reclaiming” surface mined lands – that is, follow guidelines “to conserve and develop soil, water, woodland, fish and wildlife, recreation sources and agricultural productivity.”

Although implementation of this law mitigated some of the environmental impacts of surface mining, such as soil erosion, water pollution, and losses of native biodiversity, the landscapes were still significantly degraded. Unfortunately, the legislation did not prohibit the immensely destructive and short-sighted coal extraction method known as mountaintop removal mining, in which the summit or top of a mountain is removed and the “overburden,” or removed vegetation, soils, and bedrock, is pushed into adjacent valleys. This approach to coal mining was used in many areas in the first decade of the 21st century in the United States. Portions of streams that were not completely buried were polluted with both metals and sulfates that leach from the overburden, degrading ecosystems, eliminating native species and harming nearby human inhabitants (Palmer *et al.*, 2010). Perhaps some lessons regarding the irrevocable damage of this method of coal extraction have been learned: the U.S. EPA recently revoked a permit that allowed expansion of one of the nation’s largest surface coal mines.

Mining has been responsible for transboundary pollution events. For example, in 2000, an accidental release of 100,000 cubic meters of heavy metal-laden cyanide solution, used to recover gold from mine tailings, escaped from a tailings dam at the Australian- and Romanian-owned Baia Mare gold mine in Romania. An estimated 40-km-long plume flowed into the Tisza River, a tributary of the Danube. Concentrations of cyanide in these rivers in Romania, Hungary, and Serbia were up to 700 times the allowable limit, killing much of the aquatic life and preventing use of river water by human residents. Both fish-eating wildlife and the local fishing industry were deprived of their essential catch. Fortunately, although cyanide is highly toxic, it does not persist very long in the environment. In addition, large volumes of copper, lead, and zinc were also released into the rivers. Unfortunately, the long history of environmental contamination of the rivers made it difficult to isolate the specific damage induced by the Baia Mare incident. Due to this incident, other mining disasters, and its lax environmental laws related to mining and the cyanide-extraction method, Romania was harshly criticized by its downstream neighbors.

Smelting, the process of removing metals from ore, results in deposition of aluminum, cadmium, fluoride, lead, zinc, and/or other elements that are highly toxic to plants, soil-dwelling invertebrates, and vertebrates. Such toxicity can reduce the diversity of flora and soil fauna, leaving only more tolerant species so that ecological function in contaminated landscapes is altered for centuries and likely millennia. An example is provided by two zinc smelters that operated near Palmerton in the mountains of eastern Pennsylvania for much of the 20th century. Approximately 485 ha of vegetation was severely degraded by the contaminants. Ecological

damage extended many kilometers downwind of the smelters, including well beyond the areas of obvious impact (Beyer and Storm, 1995). Areas that remained forested had fewer species of moss and lichen, and surviving trees were mainly species that resprouted from roots or stumps instead of seeds (Oyler, 1988). Retardation of decomposition due to greatly reduced populations of soil and litter macro- and microorganisms resulted in a buildup of plant litter on top of mineral soil (Strojan, 1978). Drought and fire, followed by erosion of as much as 30–60 cm of topsoil, exacerbated the damage to the mountainsides. These habitat changes and toxic levels of cadmium, lead, and zinc were associated with reduced populations of forest birds, small mammals, and salamanders, especially insectivorous species. Forest floor salamanders were extirpated. The sheer volume of contaminated soil, along with a cinder bank or “dross” pile containing an estimated 33 million tons of slag (waste ore), makes removal of contaminated material impractical. In the 1990s, to control the pH, prevent further erosion, and provide a medium for revegetation, mixtures of fly ash, sewage sludge, and limestone were applied to the barren mountainsides. Such efforts on the steep, rocky, highly contaminated slopes have been largely unsuccessful to date (Oyler, 1988).

Natural waters and soils can sometimes harbor toxic concentrations of salts and trace elements. For example, arsenic and selenium can be present at levels of concern in ancient groundwater, and pumping from wells for drinking and irrigation makes them available at the surface. Also, whereas most natural soils contain low concentrations of toxic elements, soils in some areas are naturally high enough to result in toxic effects in wildlife. Important examples exist in the western United States. Due to the marine origins of the adjacent mountains, the soils of the San Joaquin Valley in California are high in salts and certain trace elements, including selenium. Irrigation is necessary to leach and wash away salts from the soil to allow the production of vegetables and other crops. Agricultural wastewater is then carried away by a network of canals. In the past, the water was directed to a basin that formed 12 constructed wetlands on what became the Kesterson National Wildlife Refuge. These wetlands, located in an arid region with a diminished wetland base, provided an attractive habitat to a variety of breeding waterbirds. However, high concentrations of selenium in wastewater built up in the food webs of these wetlands, in part because of the rapid evaporation of water in the arid climate of central California. By 1983, selenium toxicosis had resulted in a high incidence of embryo mortality and deformities, including reduced or missing eyes and beaks, and clubbed limbs in wetland birds such as grebes, coots, stilts, and ducks (Ohlendorf and Hothem, 1995). Because of the severe impacts of selenium pollution, the ponds



were closed and filled. Subsequently, 12 other water reclamation projects in the arid western United States were also found to be contaminated by selenium from irrigation drainage water. Although this issue has received attention from planners and researchers, and some remediation has been undertaken, the underlying issues are socioeconomically, politically, and technically complex, and progress in finding solutions has been slow.

In addition to the impacts of effluents from mining related to a lack of, or poor enforcement of, environmental protection laws, the pursuit of minerals may also lead to armed conflicts and human rights abuses ([United Nations Department of Public Information, 2001](#)). For example, conflict diamonds (i.e., diamonds that “originate from areas controlled by forces or factions opposed to legitimate and internationally recognized governments, and are used to fund military action in opposition to those governments, or in contravention of the decisions of the United Nations Security Council”) have fueled prolonged civil wars, environmental devastation, and human suffering. Affected areas in West Africa include parts of Angola, Sierra Leone, and Liberia.

Throughout the world, mining companies have often been set up and operated in a manner so that they can, by design, go bankrupt when mineral deposits are depleted, thereby foregoing the costs of environmental rehabilitation. Recognizing the harm to human populations, wildlife, and ecosystems related to mismanaged mineral exploitation, not only by mining companies but also by small-scale artisanal workers, societies now have the option to educate their citizens and to alter laws, regulations, incentives, disincentives, and enforcement procedures to create safer and more productive environments. In an era of globalization of economies, the only rational course to protect people, animals, and ecosystems is to counter the “race-to-the-bottom” through globally enforced standards for coordinated environmental and human rights stewardship. Developing international standards to control problems associated with mining in the areas where the minerals are found throughout the world should be a top priority ([Hilson, 2003](#)).

## OXIDES OF NITROGEN AND SULFUR AND THEIR INTERACTIONS WITH METALS

The world is becoming more acidified ([Galloway, 2001](#)). In the anaerobic environments of sediments and geologic deposits, reducing chemistries and microbes, such as sulfate-reducing bacteria, have produced massive amounts of sulfides. However, mining and other forms of exposure of these deposits to air allow for oxidation to form

sulfur oxides and sulfates. Also, burning of coal rapidly converts sulfides to sulfur dioxide. Sulfur dioxide is also produced by refining and burning of oil and natural gas. Nitrogen, as  $N_2$  gas, comprises almost 80% of the Earth's atmosphere, and  $N_2$  is oxidized to form nitrogen oxides ( $NO_x$ ) during burning of gasoline, diesel fuel, and coal. When sulfur oxides ( $SO_x$ ) and  $NO_x$  combine with water vapor and precipitation, the resulting sulfuric, nitric, and nitrous acids can build up in the environment, leading to harmful reductions in the pH of soils and water bodies.

Coal-fired power plants in the United Kingdom have contaminated the environments of Sweden, Norway, and eastern Europe with acid deposition. Likewise, similar sources in the midwestern and eastern United States have fed air masses that delivered huge amounts of sulfur dioxide and acids to New England and Canada. Acidification is especially impactful in poorly buffered water bodies (e.g., water bodies with granite bottoms, in contrast to bottoms of limestone, which consists largely of the potent buffer calcium carbonate). Such granite bottom areas are common in the northeastern United States and areas of Europe. Adverse effects of acidification in lakes and forests in New England, eastern Canada, and Europe are well documented. One grave impact of acidification is the reduced capacity of invertebrates to precipitate calcium carbonate to form and harden their shells.

The acids also change soil and water chemistry, solubilizing ubiquitous aluminum (the third most abundant element in the Earth's crust). Then, when an acidified stream merges with a water body of a higher pH, the aluminum (as well as other metals such as iron) can readily precipitate on the gills of fishes as well as aquatic invertebrates, preventing gas exchange ([Glynn \*et al.\*, 1992](#); [Soucek, 2006](#)). Highly acidic waters with excess aluminum can also cause sludging of blood in aquatic organisms and reduced calcium uptake via gills. As a result, slowed growth, delayed development, and increased death losses are found in exposed aquatic vertebrates and invertebrates. Technologies to reduce contamination by  $SO_x$  and  $NO_x$  are widely used in the developed world, and acid deposition is less of a concern than in the past. However, considering the rapid exploitation of coal and other fossil fuels throughout the world, and especially in developing countries, a strong global effort to employ scrubbers and other technologies, and thus to prevent acidification and protect aquatic and terrestrial ecosystem resources, is needed.

## RADIATION/RADIONUCLIDES

The nuclear age began with the discovery of X-rays in 1895. Fission was first demonstrated in 1938 by German

scientists, which led to competition between Nazi Germany and the United States to develop an atomic weapon. The United States developed such a bomb in 1945 and then used two against Japan to end World War II. Nuclear weapons testing and the bombing of Hiroshima and Nagasaki, Japan, during the mid-20th century resulted in the global distribution of radioactive material.

The first reactor-produced radioisotopes for medical and industrial use were provided by the Oak Ridge National Laboratory in 1946. Since that time, nuclear medicine has been a boon for the diagnosis and treatment of many diseases.

By 1951, electricity was being produced via nuclear fission, and by July 1957, the first civilian reactor for power was online. However, in October 1957, the first accidental release of radiation from a reactor occurred. Theoretically, nuclear energy can provide cleaner and cheaper electricity than burning of fossil fuels. However, in addition to the high expense of construction and maintenance, and the small releases of radioactivity during normal operations as well as concerns regarding the transportation and storage of nuclear wastes, there have been accidental discharges of larger amounts of radiation, such as the incidents at Three Mile Island, Pennsylvania, in 1979 and most notably at Chernobyl, Ukraine, in 1986 and Fukushima, Japan, in 2011. The most recent incident was the consequence of a combination of poor choice of location, a poor design, and resultant flooding immediately after an earthquake and an immense tsunami. Currently, the Fukushima accident remains to be resolved, and retired employees are working and sacrificing their health to help alleviate great risks to younger workers, many of whom have already been heavily exposed to radiation.

In 1986, the explosion and fire at a poorly designed reactor at Chernobyl led to the hospitalization of 203 people and the death of 47 due to radiation poisoning. Also, as of 2000, 1800 cases of thyroid cancer in children of the area had been diagnosed and more were anticipated. Fallout containing short- and long-lived radioisotopes covered a wide area of northeastern Europe, and radiation from this incident ultimately was transported throughout the Northern Hemisphere. Residents of a 30-km radius were evacuated, and few have returned. One source estimated that there would be an eventual 4000 additional human deaths, driven by a 2% increase in cancer rates. Whereas the environmental half-life of radioactive iodine (I-131) from reactors is only approximately 8 days, that of strontium (Sr-90) is approximately 29 years and that of radiocesium (Cs-137) is approximately 30 years. Hence, in the area around Chernobyl, as of 2011, less than one half-life of the latter two radioactive isotopes has transpired.

In the 30-mile exclusion zone around Chernobyl, despite somatic and germ cell mutations in birds, small

mammals, and fish associated with increased aberrant phenotypic traits, native plants and wildlife have flourished, and invasive exotic species have declined. This has been touted as “proof” that effects of radiation from this nuclear disaster were not as long-lasting as feared. However, these animals could represent the immigration of dispersing animals from surrounding areas into unoccupied habitats that are now relatively undisturbed by humans. Few detailed, longitudinal studies of wildlife populations and their age structures have been conducted. Perhaps best studied in the Chernobyl area have been barn swallows, which are migratory insectivorous birds with a high degree of philopatry. Fourteen years after the incident, swallows nesting in the area around Chernobyl had a high prevalence of abnormal sperm and reductions in antioxidant levels compared to swallows from a reference area. The exposed birds also had a sustained high rate of partial albinism and germline mutation rates that were 2- to 10-fold higher than those of birds at distant reference sites. The mutations likely accounted for immune suppression, reduced carotenoid-based sexual coloration, and abnormal feather lengths (defective phenotypic secondary sexual traits), which may undermine reproductive success (Møller *et al.*, 2004). Small mammal populations in some areas near Chernobyl were reduced by as much as 90% following the reactor incident. Increases in populations the following spring were attributed to immigration from less contaminated adjacent areas and the cessation of tillage and crop harvest (Sokolov *et al.*, 1993). Voles collected from the Chernobyl area had the highest radiocesium burdens and dose rates ever recorded for mammals. Researchers have reported high genetic diversity in voles from the highly contaminated zone, which could be indicative of high mutation rates and/or immigration from less contaminated areas. Cytogenetic damage was also documented in voles in Belarus and Sweden, both of which were in the direction of prevailing winds in the first few days following the disaster (Goncharova and Ryabokon, 1995).

## THE FUTURE OF ECOTOXICOLOGY

Trends in wildlife numbers are telling. The World Wildlife Fund's Living Planet Index estimates that the number of free-ranging wild vertebrates on Earth declined by approximately 30% from 1970 to 2007 ([http://www.panda.org/about\\_our\\_earth/all\\_publications/living\\_planet\\_report/living\\_planet\\_report\\_graphics/lpi\\_interactive](http://www.panda.org/about_our_earth/all_publications/living_planet_report/living_planet_report_graphics/lpi_interactive)). Although gains were seen in temperate climates of the north, countries of the tropics and the Southern Hemisphere have shown overwhelming declines. Accordingly, across the globe, our vertebrate relatives are being devastated.

Unlike domestic animals and most people, many wild animals face extreme challenges daily and sometimes many times a day. Differences in milliseconds of response time can determine the likelihood of successful predation as well as whether animals avoid becoming prey. Also, females of many species will breed only with males that display ideal conformation and behaviors. Moreover, organisms in the wild are routinely confronted with a host of potential pathogens: their water is never filtered or disinfected, and their food is never dried, preserved, or cooked. Although a high plane of health is essential for wild animals to contribute to future generations in the wild, there is no disputing that their health is being undermined not only by habitat loss, climate change, exotic species introductions, overharvest and pathogen pollution, but also by a host of chemical contaminants – both man-made and produced by toxigenic microbes, plankton, and plants. If various forms of environmental mismanagement continue to place greater numbers of species at risk of extinction, as seems likely, attention to toxic effects on individuals will be increasingly necessary. Moreover, ethically consistent animal health professionals, manufacturers, the public, and the regulatory community cannot ignore the suffering and death losses of wild animals any more than they would ignore suffering and deaths in publically or privately held animals within laboratories, homes, or farms. Tolerating widespread poisoning of wildlife, when we know quite well how to prevent toxic exposures, is irresponsible.

In the view of the authors, ecotoxicology should always be a balance of ecological, biomedical, and chemical sciences. Ecotoxicology initially was focused largely on determining chemical residues in the environment, and later it focused more on mechanisms and effects of select groups of toxicants. To be most effective, ecotoxicological research should include both aspects. Ecotoxicology should also include assessment of effects on individuals, populations, biotic communities, and ecosystems. Now and in the future, ecotoxicology should strive to reach a point where it has been so successful that remediation of contaminated sites is no longer necessary. Thereafter, future efforts could relate almost exclusively to prevention of poisoning. Currently, achieving that goal is difficult, and it is becoming more so in much of the developing world.

Although diagnostic laboratories should become involved in forensic ecotoxicology on a daily basis, simply waiting for specimens to be brought in for studies will fail to provide adequate protection of wildlife and ecosystems. Thus, diagnostic and forensic ecotoxicology must become a routine, proactive, collaborative enterprise involving trips to the field for monitoring of community structure, population trends, exposures, and impacts; trips to the laboratory for exploratory and confirmatory research; and common interactions

with manufacturers, end users, wildlife managers, and regulatory agencies to ensure responsible chemical stewardship.

The authors believe that there is a need for better testing of products before marketing to ensure not only the health of humans but also that of pets, livestock, wildlife, plants, and the environment as a whole. Products already on the market but not thoroughly investigated to date should be reexamined. To enhance diagnostic and forensic capabilities, studies designed to identify causes and effects should examine test organisms via physical examinations; clinical pathology assays; gross and histologic pathology assessments; assessment of genomic, proteomic, and metabolomic impacts; and chemical analyses to determine residues. These endpoints should be evaluated at doses with no impact, threshold toxic reactions, marked toxic reactions, and overt lethal toxicity.

For most toxicants, detailed and comprehensive ecotoxicological information exists for only a relatively very few plant and animal species. Risk assessments rely on extrapolation from these surrogate species to the huge array of organisms that will ultimately be exposed when the product is used or the effluent is released. Without requirements for effective monitoring and reporting on the effects of exposures of a host of biota in the field under conditions after the introduction of contaminants to the environment, toxic effects are bound to be missed. Accordingly, postmarketing studies of impacts on native organisms should be a new priority.

Of concern is that much information from industry on testing of products is buried in reports to the U.S. EPA and difficult to obtain. We contend that upon granting of patents, industry scientists should be required to publish results of toxicity testing in the scientific literature and via the Internet so that the information is fully available to scientists and the interested public. Stakeholders aware of toxicological data should share in decision making with regard to risk management and longer term product registration. We are also of the view that considerable effort in regulatory ecotoxicology should be devoted to removal of problematic compounds and formulations, with replacement as warranted, with products unlikely to present undue ecological harm.

Because of the reality of modern-day exposures, far greater attention needs to be devoted to complex mixtures of chemicals, the fate and toxicity of chemical degradation products, the indirect effects of contaminants, and the validation of risk assessments. These deficits indicate a need for more integrative ecotoxicology research, including comprehensive, large-scale field studies examining interactions among biotic and abiotic factors, complemented by microcosm and mesocosm studies. Also, more research incorporating reproductive toxicology and multigenerational studies is needed. In addition, more studies should examine how contaminant

exposure affects animal behavior because subtle changes in behavior can have immense negative impacts on an animal's survival and reproductive fitness.

Because of the scale and complexity of challenges in ecotoxicology, filling critical data gaps and implementing adaptive management actions can most often be achieved as a member of a collaborative team. Single individuals in such teams may "wear more than one hat" in this process. In any case, expertise is often needed in ecology, wildlife biology, wildlife management, veterinary medicine, veterinary toxicology, ecotoxicology, analytical toxicology, epidemiology, pathology, immunology, environmental risk assessment, environmental law and policy, ecological rehabilitation, and other areas. To get under way and to help ensure a recognized positive impact, individual scientists and groups may choose to focus their attention by specializing in a particular toxicant group (e.g., metals, herbicides, or hazardous wastes), an animal species group (e.g., marine mammals, birds, or invertebrates), a given habitat type or biome (e.g., deserts, forests, mountains, tundras, estuaries, or coral reefs), or a given region (e.g., the midwestern United States, north Africa, tropical Asia, or Antarctica).

The prospects for a healthy environment for humans, domestic animals, and wild biota depend to a large extent on whether we learn to prevent poisoning and ecological disruption by naturally occurring and synthetic chemicals. In the world of tomorrow, rather than relying on more fertilizers, more pesticides, and more raw materials, food must be grown in new ways and in new places, with only benign chemical inputs and closure of nutrient loops. To conserve energy, production of food should be closer to consumers, but to reduce the adverse impacts of the agricultural footprint, production of food should also take place in what is currently unused space such as in open oceans and barren deserts in concert with astute forms of desalination of seawater. Other products and services must also be provided more efficiently and cleanly. Clean and renewable energy sources, cradle-to-cradle technologies, and other innovations offer the option to move strongly in the direction of rehabilitating ecosystems to achieve recoveries of biodiversity and improved health. These are not someone else's problems. They are our problems, and toxicologists, practitioners, and other health professionals of many stripes have unique and timely opportunities to lead.

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## Avian toxicology

*Robert H. Poppenga and Snehal Tawde*

### INTRODUCTION

There are approximately 30 orders of birds in the Aves class, with approximately 2000 genera and 10,000 species. Obviously, such a large class of animals possesses a bewildering array of unique physiologic adaptations, behaviors, and ecologic niches. Thus, a discussion of a broad topic such as avian toxicology presents a daunting task. This chapter provides an overview of toxicologic hazards to three subsets of birds: pet and aviary birds, poultry and other farm-raised birds, and wild birds. Given the breadth of the topic, this chapter is not an exhaustive treatise but, it is hoped, provides an overview of major avian toxicologic hazards.

#### Pet and aviary birds

The popularity of birds as pets has increased substantially during the past several years. The increased popularity is due to a variety of factors, including the availability of species with a diversity of sizes, colors, and temperaments; the ability to house birds in a small area; and, in some cases, a long life span. The fact that most pet birds are confined to the home environment exposes them to toxicants that poultry and wild birds are unlikely to come into contact with, such as the pyrolysis products from Teflon-coated cookware. Alternatively, pet birds can be exposed to toxicants to which poultry and wild birds are also exposed but that are in different forms or from different sources. For example, pet birds can be intoxicated by lead, most often in the form of

lead-based paint or lead objects such as drapery weights or toys, whereas wild birds are frequently intoxicated following ingestion of lead ammunition or lead-containing fishing gear.

#### Wild birds

Obviously, wild birds exist in a much less well-controlled environment than do pet birds or poultry and are therefore exposed to a greater variety of potential toxicants. It is safe to say that avian toxicology had its origins in the 1950s and 1960s when the effects of organochlorine insecticides (OCs) such as dichlorodiphenyltrichloroethane (DDT) on a variety of bird species were first recognized. The rapid decline of a variety of high-profile avian species such as bald eagles, peregrine falcons, and pelicans during those years and, more recently, the declines of many songbird and waterfowl populations have resulted in significant government funding to more fully characterize the effects of toxicants on avian wildlife. In addition, regulatory requirements to obtain premarket toxicology information for pesticides using laboratory surrogates, such as quail and mallard ducks, have resulted in the generation of a substantial database of toxicologic information.

One unique aspect of wild bird toxicology is the potential exposure of wild birds to toxicants via their prey. Raptors and scavengers such as turkey vultures or condors are often poisoned as a result of feeding on animal carcasses contaminated with pesticides or lead. Thus, secondary or relay toxicosis is a more common occurrence in wild birds than in pet birds or poultry.



## Poultry

Most commercial poultry are raised in well-controlled environments and are provided quality feed and water. Thus, their potential for exposure to toxicants is more limited. However, poultry can become intoxicated following exposure to common feed additives such as organic arsenicals or ionophores if feed misformulations occur. Obviously, free-ranging poultry are potentially exposed to a greater variety of hazardous chemicals.

## Physiologic differences of birds relevant to toxicology

### Respiratory system

The unique physiology of bird respiration makes them more susceptible to some inhaled toxicants, such as carbon monoxide (CO) and pyrolysis products from overheated Teflon. The sensitivity of birds to inhaled toxicants brings to mind the image of the canary in the coal mine. Several physiologic differences of birds increase their sensitivity to inhaled toxicants, such as a higher mass specific minute ventilation, a higher mass specific ventilation of gas-exchange tissues, cross-current and countercurrent gas exchange mechanisms, and a gas diffusion barrier one-half the thickness of that of mammals (Brown *et al.*, 1997). Some of these physiologic adaptations are a result of high metabolic rates of birds and the concomitant need for a high ventilatory capacity.

### Metabolic system

A number of studies have examined the metabolism of xenobiotics in a variety of avian species and compared metabolic capabilities of birds and mammals (Pan, 1978; Dalvi *et al.*, 1987). Xenobiotic metabolism has been studied most extensively in chickens, pigeons, Japanese quail, and domestic ducks (Pan, 1978). As expected, there are substantial differences in metabolic pathways and capacities between birds and mammals that influence susceptibility to intoxication. Organophosphate (OP) insecticides are more toxic to birds than mammals, and the basis for this difference is primarily due to differences in OP metabolism. Dimethoate is 20 times more toxic for pheasants than for rats. Dimethoate is metabolized by pheasants to a toxic metabolite, whereas the toxic metabolite is detoxified by rats (Pan, 1978). In addition, there are differences between bird species and between sexes and ages. Geese, chickens, and turkeys had similar liver aniline hydroxylase activity compared with that of rats, whereas quail and ducks had lower activities (Dalvi *et al.*, 1987). Conjugating capabilities also differ. For example, glucuronidation of *p*-nitrophenol was greater in ducks than in chickens or turkeys.

### Excretion

Birds have several unique excretory pathways for xenobiotics. Many metals are incorporated into feathers, which are subsequently molted. In addition, incorporation of lipophilic toxicants into eggs provides a significant excretory pathway for female birds.

## GENERAL COMMENTS ABOUT DIAGNOSING AVIAN INTOXICATIONS

Although most diagnostic approaches for documenting intoxication in mammals are applicable to birds, there are several unique aspects that bear mentioning. Given the small size of many birds, obtaining a sufficient amount of sample for testing can sometimes present a challenge. For example, it is not unusual to be able to obtain only 100  $\mu$ L (sometimes less) of whole blood or serum antemortem for lead and zinc testing, respectively. Quantities of gastrointestinal contents or tissues such as liver are sometimes limiting, especially if multiple tests are desired. The availability of less than optimal sample sizes often decreases the sensitivity of toxicologic testing, so it is possible that low toxicant concentrations are not detected. In the case of wild bird die-offs, pooling of samples from several dead birds is often required.

Investigation of wild bird die-offs is often a challenge from the standpoint that frequently birds are not discovered until significant postmortem autolysis and/or predation has occurred. Postmortem autolysis often precludes thorough pathologic, microbiologic, and virologic testing. However, for the vast majority of toxicologic tests, the condition of the tissue or fluid sample is of less concern, and severely autolyzed samples may be perfectly suitable for toxicologic testing.

Antemortem, whole blood is most often requested from mammals for cholinesterase activity determinations. However, in birds, there is little cholinesterase activity that is associated with the red blood cell. Therefore, for avian species, plasma is preferred. Feathers and egg samples are sometimes useful for diagnosing either exposure to or intoxication from toxicants.

As is the case for any suspected intoxication related to feed, it is critical to obtain a representative feed sample. For example, feed-related mycotoxicoses are common concerns in poultry. However, the distribution and concentration of a mycotoxin in a feed can be quite variable. Therefore, it is important to obtain a number of samples that can then be pooled and subsampled. Other toxicants for which obtaining representative feed samples are critical include drugs; growth promotants such as ionophores; sodium chloride; and nutritionally important minerals such as copper, zinc, and selenium.

Because malicious poisonings of wild birds are common and some poisoned birds are protected by state or federal laws, the potential for litigation should always be of concern. Therefore, it may be critical to obtain and submit samples following chain-of-custody procedures.

## NATURAL TOXICANTS

### Algal toxins

Wild bird species are more likely to be intoxicated by cyanobacterial toxins than are pet birds or poultry given their most common water sources. However, it is possible that zoo birds or production birds such as poultry, game birds, or waterfowl can be exposed via their water depending on its source and if conditions conducive to algal blooms are present. There are few documented instances of intoxication of birds, although cyanobacterial toxins are often found in lakes, reservoirs, and rivers used by wild birds. A survey of the saline Salton Sea in California detected microcystins in 85% of the samples tested (Carmichael and Li, 2006). Analyses of liver samples of eared grebe (*Podiceps nigricollis*) found microcystins at concentrations believed to be clinically significant. However, the role that microcystins play, if any, in documented large eared grebe die-offs remains to be determined. A bloom of *Microcystis aeruginosa* in a pond in Japan was believed to have played a role in the death of approximately 20 spot-billed ducks (Matsunaga *et al.*, 1999). Although analysis for microcystins was not done, hepatic necrosis was noted in the one bird necropsied.

Anatoxin-a(s) was believed to have been the cause of bird mortality in several Danish lakes. *Anabaena lemmermannii* dominated the blooms, and anatoxin-a(s) was detected and bloom material was shown to be toxic to mice (Henriksen *et al.*, 1997; Onodera *et al.*, 1997). Both anatoxin-a and microcystins (LR and RR) were implicated in the mass mortalities of flamingos in Kenya (Krienitz *et al.*, 2003).

### Avian vacuolar myelinopathy

Avian vacuolar myelinopathy (AVM) is a disease of birds that has sporadically caused mortality of bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*), and other waterbirds in several reservoirs in the southeastern United States. AVM is characterized by widespread, bilaterally symmetrical vacuolation of the white matter of the brain and spinal cord. Work has demonstrated an association between bird mortality and the presence of the invasive plant hydrilla (*Hydrilla verticillata*) (Wilde *et al.*, 2005). An unknown cyanobacterial species belonging to *Stigonematales* has been found to be

associated with the plant. AVM was reproduced using mallard ducks exposed to *Stigonematales* blooms. It is believed that a currently uncharacterized algal neurotoxin is the cause of AVM.

### Plants

A number of plants are recognized for their toxicity for birds (Table 67.1). Plants contain a large variety of biologically active constituents, including volatile oils, resins, alkaloids, polysaccharides, phenols, glycosides, and fixed oils. Free-flying companion birds are likely to encounter and eat household plants. Free-ranging production birds encounter plants in their outdoor environment. Both companion birds and production birds can be exposed to potentially toxic plants via their feed. Generally, wild birds are adapted to their environments and are unlikely to inadvertently ingest toxic plants.

The susceptibility of different bird species to specific toxic plants is variable. In addition, birds may be unaffected by plants that are toxic to other animals such as mammals. For example, cedar waxwings and house finches can consume fruit from the pepper tree (*Capsicum annuum*) that is toxic to mammals (Navarro, 1992).

Feeding behaviors also influence the susceptibility to intoxication. For example, it has been suggested that parrots can consume otherwise toxic plants because they remove the outer covering of fruits and seeds, which can contain high concentrations of toxins, before consumption.

There are few documented cases of bird intoxication following plant ingestion. However, there are several experimental studies of the susceptibility of small companion birds such as budgerigars and canaries along with numerous feeding studies using chickens and turkeys. There are published and anecdotal reports of fruit-eating birds being intoxicated by ethanol as a result of eating fermented fruit (Fitzgerald *et al.*, 1990).

### Mycotoxins

Independent chapters pertaining to mycotoxins are presented elsewhere in this book; therefore, select information pertaining to avian mycotoxicoses is presented in this section.

### Aflatoxins

Economic losses attributed to aflatoxin exposure in poultry often stem from reductions in growth rate, hatchability, feed efficiency, and immunocompetence. These naturally occurring mycotoxins are secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus*, and

TABLE 67.1 Plants reported to be toxic to birds

Common name	Scientific name	Toxin	Plant part demonstrated to be toxic	Mechanism of toxic action	Primary organ system affected	Species/bird type reported to be affected	References
Avocado	<i>Persea americana</i>	Persin	Fruit, leaves	Unknown	Cardiovascular system	Budgerigars ( <i>Melopsittacus undulates</i> ), canaries ( <i>Serinus canaria</i> ), cockatiels, ostriches ( <i>Struthio camelus</i> )	Hargis <i>et al.</i> (1989), Shropshire <i>et al.</i> (1992), Burger <i>et al.</i> (1994), Burrows and Tyrll (2001)
Black locust	<i>Robinia pseudoacacia</i>	Unknown; possibly glycoprotein lectins, proteins	Leaves, bark	Unknown	Cardiovascular system, gastrointestinal tract	Budgerigars ( <i>Melopsittacus undulates</i> )	Shropshire <i>et al.</i> (1992), Burrows and Tyrll (2001)
Bladder pod, rattlebox, coffeeweed, daubentonia, others	<i>Sesbania</i> spp.	Saponins, others	Seeds	Smooth muscle inhibition, vasodilation	Nervous system, gastrointestinal tract, kidneys	Chickens	Flory and Hebert (1984), Burrows and Tyrll (2001)
Cacao	<i>Theobroma cacao</i>	Theobromine, caffeine	Seeds	Adenosine receptor antagonism, increased intracellular Ca <sup>2+</sup> concentrations	Nervous system, cardiovascular system	Chickens	Day and Dilworth (1984)
Canola (also referred to as rapeseed), mustards	<i>Brassica</i> spp.	Glucosinolates, others	Seeds	Accumulation of trimethylamine affects eggs (odor and taste); liver and muscle damage due to unknown mechanism	Eggs, liver, muscle, cardiovascular system	Chickens, turkeys, ducks	Ratanasethkul <i>et al.</i> (1976), Wight <i>et al.</i> (1987), Burrows and Tyrll (2001)
Cassava	<i>Manihot</i> spp.	Cyanide	Root	See section on cyanide			
Castor bean	<i>Ricinus communis</i>	Ricin	Seeds	Protein synthesis inhibition	Gastrointestinal tract, liver	Ducks	Jensen and Allen (1981)
Coffee senna, sickle pod, coffeeweed, others	<i>Senna</i> spp. (previously <i>Cassia</i> spp.)	Anthraquinones	Fruit, seeds	Irritation, mitochondrial myopathy	Gastrointestinal tract, skeletal muscle	Chickens	Cavaliere <i>et al.</i> (1997), Burrows and Tyrll (2001), Haraguchi <i>et al.</i> (2003)
Corn cockle	<i>Agrostemma githago</i>	Saponins	Seeds	Irritation	Gastrointestinal tract	Chickens	Heuser and Schumacher (1942), Burrows and Tyrll (2001)
Cottonseed	<i>Gossypium</i> spp.	Gossypol	Seeds	Unknown	Gastrointestinal tract, kidneys, liver, egg production	Poultry	Brown and Julian (2003)
Coyotillo	<i>Karwinskia humboldtiana</i>	Anthracenones	Seeds	Uncoupling of oxidative phosphorylation, inhibition of axoplasmal transport	Nervous system	Poultry	Burrows and Tyrll (2001)
Day jessamine, day cestrum, wild jasmine, Chinese inkberry	<i>Cestrum diurnum</i>	Analog of 1,25-dihydroxycholecalciferol	Leaves	Hypercalcemia, dystrophic tissue calcification	Cardiovascular system, kidneys	Chickens	Sarkar <i>et al.</i> (1981)

(Continued)

TABLE 67.1 (Continued)

Common name	Scientific name	Toxin	Plant part demonstrated to be toxic	Mechanism of toxic action	Primary organ system affected	Species/bird type reported to be affected	References
Death camas, others	<i>Zygadenus</i> spp.	Alkaloids	Seeds, stems, roots	Increase reflex activity, stimulate afferent pathway receptors, increase and extend negative afterpotentials	Nervous system	Poultry	Burrows and Tyrl (2001)
Dieffenbachia	<i>Dieffenbachia</i> spp.	Insoluble oxalates	Leaves	Physical irritation	Gastrointestinal tract	Canaries ( <i>Serinus canaria</i> )	Arai <i>et al.</i> (1992)
Digitalis	<i>Digitalis purpurea</i>	Cardiac glycosides	Leaves	Inhibit $\text{Na}^+ - \text{K}^+$ ATPase	Cardiovascular system	Canaries ( <i>Serinus canaria</i> )	Arai <i>et al.</i> (1992)
Eucalyptus	<i>Eucalyptus cladocalyx</i>	Cyanide	Leaves	Inhibit cytochrome oxidase <i>c</i>	Multiorgan	Poultry	Brown and Julian (2003)
Jimsonweed, thorn apple	<i>Datura</i> spp.	Tropane alkaloids	Seeds	Cholinergic (muscarinic) receptor antagonism	Nervous system	Chickens	Day and Dilworth (1984), Kovatsis <i>et al.</i> (1994), Burrows and Tyrl (2001)
Joboba	<i>Simmondsia chinensis</i>	Simmondsin	Seeds	Inhibition of appetite via unknown mechanism	Overall growth rate	Chickens	Arnouts <i>et al.</i> (1993)
White popinac, lead tree, guacis, jumby bean	<i>Leucaena leucocephala</i>	Mimosine	Seeds	Inhibit reactions requiring pyridoxine, inhibit DNA and RNA synthesis	Bone, depressed growth	Poultry	Kamada <i>et al.</i> (1998), Burrows and Tyrl (2001)
Lily of the valley	<i>Convallaria majalis</i>	Cardenolides	Leaves, rhizomes, roots	Inhibit $\text{Na}^+ - \text{K}^+$ ATPase	Cardiovascular system	Poultry	Burrows and Tyrl (2001)
Lupine	<i>Lupinus</i> spp.	Quinolizidine alkaloids	Leaves	Cholinergic receptor agonists	Nervous system	Canaries ( <i>Serinus canaria</i> )	Arai <i>et al.</i> (1992), Burrows and Tyrl (2001)
Milkweed	<i>Asclepias</i> spp.	Cardenolides, uncharacterized neurotoxin	Entire plant, latex sap	Inhibit $\text{Na}^+ - \text{K}^+$ ATPase, uncertain for neurotoxic effect	Cardiovascular system, nervous system		Burrows and Tyrl (2001)
Nightshades, potato	<i>Solanum</i> spp.	Steroidal glycosides (chaconine, solanine, others)	Immature seeds, foliage	Saponin-induced irritation, AChE inhibition	Gastrointestinal tract, nervous system	Chickens	Temperton (1944), Burrows and Tyrl (2001)
Oak	<i>Quercus</i> spp.	Tannins	Leaves, early buds	Astringent effect (protein precipitation), direct cell damage	Gastrointestinal tract, kidneys ( <i>Casuaris casuaris</i> )	Double-wattled cassowary	Kinde (1988), Burrows and Tyrl (2001)
Oleander	<i>Nerium oleander</i>	Oleandrin	Leaves	Inhibit $\text{Na}^+ - \text{K}^+$ ATPase	Cardiovascular system	Budgerigars ( <i>Melopsittacus undulates</i> ), canaries ( <i>Serinus canaria</i> ), geese (species not identified)	Arai <i>et al.</i> (1992), Shropshire <i>et al.</i> (1992), Alfonso <i>et al.</i> (1994)



Oxalates, soluble	Many plants ( <i>Halogeton</i> spp., <i>Sarcobatus</i> spp., others)	Calcium or potassium oxalates	Leaves, stems	Hypocalcemia, calcium oxalate precipitation in renal tubules	Cardiovascular system, kidneys	Poultry	Burrows and Tyrl (2001)
Parsley	<i>Ammi majus</i>	Furanocoumarins	Seeds, leaves	Primary photosensitization, other (calcium channel inhibition)	Skin, liver, kidneys	Ducks, geese; chickens and turkeys are less sensitive	Shlosberg <i>et al.</i> (1974), Burrows and Tyrl (2001)
Poison hemlock	<i>Conium maculatum</i>	Pyridine alkaloids including coniine, <i>N</i> -methylconiine, $\gamma$ -coniceine	Seeds	Nicotinic receptor agonist	Nervous system	Range turkeys (species not identified)	Frank and Reed (1987), Burrows and Tyrl (2001)
Pokeberry	<i>Phytolacca americana</i>	Saponins, oxalates	Berries	Irritation	Gastrointestinal tract	Turkeys	Cattley and Barnett (1977), Burrows and Tyrl (2001)
Rattlebox, others	<i>Crotalaria</i> spp.	Pyrrolizidine alkaloids	Seeds, leaves, stems	Inhibition of protein and RNA synthesis, alkylate DNA, inhibit mitosis	Liver	Chickens, geese	Gopinath and Ford (1977), Alfonso and Sanchez (1993), Burrows and Tyrl (2001)
Ragwort, groundsel	<i>Senecio</i> spp.	Amino acids, nitriles, $\beta$ -aminopropionitrile	Seeds	Glutamate receptor agonism, inhibition of lysyl oxidase	Nervous system, reproduction (decrease ovarian activity), eggs	Chickens, turkeys	Chowdhury (1988), Burrows and Tyrl (2001)
Sweet pea	<i>Lathyrus</i> spp.						
Tobacco	<i>Nicotiana</i> spp.	Nicotine, nornicotine, anabasine	Leaves and stems	Nicotinic receptor stimulation, teratogen	Nervous system, embryo development	Poultry	Burrows and Tyrl (2001)
Velvetweed	<i>Abutilon theophrasti</i>	Cyanogenic glycosides (probably not toxic), $\beta$ -cyano-L-alanine	Seeds	Affects egg yolks due to unknown mechanism	Eggs	Poultry	Brown and Julian (2003)
Vetch	<i>Vicia</i> spp.			Cyanide inhibits cytochrome oxidase <i>c</i> ; signs similar to pyridoxine deficiency	Nervous system	Chickens	Harper and Arscott (1962), Burrows and Tyrl (2001)
Virginia creeper	<i>Parthenocissus quinquefolia</i>		Leaves	Unknown	Gastrointestinal system	Budgerigars ( <i>Melopsittacus undulatus</i> )	Shropshire <i>et al.</i> (1992), Burrows and Tyrl (2001)
Yew	<i>Taxus</i> spp.	Taxine	Leaves, seeds	Alter atrioventricular conduction via inhibition of $\text{Na}^+ - \text{Ca}^{2+}$ fluxes and possible $\text{K}^+$ channel effects	Cardiovascular system	Budgerigars ( <i>Melopsittacus undulatus</i> ), canaries ( <i>Serinus canaria</i> ), emus ( <i>Dromaius novaehollandiae</i> )	Shropshire <i>et al.</i> (1992), Fiedler and Perron (1994), Burrows and Tyrl (2001)

*A. nomius*. In 1959, the infamous turkey "X" disease, involving the deaths more than 100,000 turkey poults and ducklings in England, was determined to be due to multiple aflatoxins, including cyclopiazonic acid, in imported Brazilian peanut meal fed to affected birds (Cole, 1986). Structurally, aflatoxins (AFs) are difurocoumarin derivatives that fluoresce under ultraviolet light. Depending on the color of the fluorescence, AFs are divided into aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and AFB<sub>2</sub> for blue and AFG<sub>1</sub> and AFG<sub>2</sub> for green (Dalvi, 1986). Although peanuts and corn are commonly known substrates for the growth of aflatoxin-producing fungi, other grains and nuts have been implicated as well. The U.S. Food and Drug Administration (USFDA) has set a current guidance level for AFB<sub>1</sub> concentrations in corn and peanut products intended for immature and mature poultry at 20 and 100 parts per billion (ppb), respectively. Moisture content greater than 14%, relative humidity greater than 70%, and temperature greater than 70°F are often conducive for the growth of *Aspergillus* sp. fungi in improperly stored grain. Fungal invasion of field crops, especially during drought stress or when there is insect or mechanical damage, can occur.

Mortality in cranes and waterfowl due to consumption of waste grain in standing crops containing acutely toxic aflatoxin concentrations has been reported, coinciding with migration and wintering. Contaminated grain offered at bird feeding stations, however, can lead to clinical signs at any time of the year. Exposure to aflatoxins and subsequent mortality events have been reported in free-ranging birds, including a variety of duck species (mallard, black duck, lesser scaup, gadwall, and blue- and green-winged teal), Canada geese, snow geese, and sandhill cranes (Friend and Franson, 1999).

Poultry, especially turkeys, are extremely sensitive to the toxic effects of AFB<sub>1</sub>, with quails being intermediate and chickens being comparatively resistant (Lozano and Diaz, 2006). AFB<sub>1</sub> at 0.7 mg/kg reduced the growth rate of turkey poults but had no effect in quails and chickens (Arafa *et al.*, 1981). Significant reduction in weight gain and feed consumption was observed in day-old broiler chicks fed 10 ppm AFB<sub>1</sub> for 8 weeks, especially after the third week (Dalvi and Ademoyero, 1984). In day-old ducklings, the acute toxicity for AFB<sub>1</sub> has been estimated to be 12 µg/duckling (Purchase, 1967).

Clinical signs in birds include lethargy, depression, blindness, inability to fly, tremors, and wing flapping, although birds can also simply be found dead. Lethal aflatoxicoses in ducklings occurred as inappetence, reduced growth, abnormal vocalization, feather picking, purple discoloration of leg and feet, and lameness. Ataxia, convulsions, and opisthotonus preceded death (Asplin and Carnaghan, 1961). Turkeys develop inappetence, reduced spontaneous activity, unsteady gait, recumbency, anemia, and death (Wannop, 1961). Aflatoxicosis in chickens closely resembles the clinical signs in ducks and turkeys.

Chronic effects, which include appetite loss, weight loss, and general ill health, can be more insidious and difficult to definitively relate to aflatoxin exposure. Chronic exposure can also produce a shrunken, fibrous liver with regenerative nodules or tumors. Chronic low-level aflatoxin exposure is also known to suppress the immune system, which may predispose animals to infectious diseases.

It is well recognized that several mycotoxins may act in combination to cause lesions previously considered to be consistent with aflatoxicoses; cyclopiazonic acid, sterigmatocystin, and possibly others play a role in the development of this disease. Acute exposures over a relatively short period lead to pale, swollen, and enlarged livers (and kidneys), occasionally accompanied by gallbladder distension. Hemorrhages in internal organs may be accompanied by generalized edema. The crop and proventricular mucosa shows thickening (Kumar and Balachandran, 2009). Inflammation and bleeding of the gastrointestinal (GI) tract lining, which may cause the intestines to appear blackish-red throughout their entire length, can also be observed. Microscopically, the hepatocytes show fatty changes, proliferation of bile ductules, and extensive fibrosis, accompanied by vascular and degenerative lesions in pancreas and kidney (Hoerr, 1997).

Measurement of aflatoxin concentrations in suspected feed, ingesta, or the liver is crucial for confirming the diagnosis. Representative sampling and adequate precautions (e.g., freezing samples) to avoid fungal growth and toxin production secondary to improper post-collection storage and shipping are necessary.

Suspected feed sources should be removed from the diet. Selenium supplementation has been suggested to have some protective action in Japanese quail fed 1 ppm (mg/kg) AFB<sub>1</sub>. Field crops frequented by avian wildlife for seasonal feeding may be monitored for aflatoxins, especially during hot and humid weather. At concentrations of 100 ppb and higher, deep plowing of fields containing the contaminated grains is recommended by the U.S. Geological Survey National Wildlife Health Center (Friend and Franson, 1999). If that is not possible, hazing the birds away may be another strategy.

Aflatoxin contamination of feedstuff is nearly universal, and chemopreventive and/or enterosorptive approaches have been attempted. Synthetic zeolites incorporated at 1% in diet were observed to be protective against 2.5 mg/kg AFB<sub>1</sub> in feed (Miazzo *et al.*, 2000). Hydrated sodium calcium aluminosilicate, fed at dietary levels ranging from 0.25 to 1%, has been shown to diminish the deleterious effects of AFs (up to 5 mg/kg in diet) in broiler chicks (Kubena *et al.*, 1998; Ledoux *et al.*, 1999). Silymarin, a hepatoprotective agent used in humans, may be useful in the treatment of aflatoxicosis due to promotion of cellular synthesis of macromolecules, antioxidant activity, and stabilization of cell membranes (Tedesco *et al.*, 2004). Dietary

inclusion of the food antioxidant butylated hydroxytolouene at a level of 0.39% ameliorated weight loss in chickens consuming AFB<sub>1</sub> at 3 mg/kg in their diet (Larsen *et al.*, 1985) due to an inhibitory effect on the activity of Cyt P450 1A enzymes, suggesting reduced bioactivation of AFB<sub>1</sub> to the AFBO (Klein *et al.*, 2003). *Lactobacillus rhamnosus* GG was able to bind AFB<sub>1</sub> in adapted Caco-2 cells and thereby reduce its transport, metabolism, and toxicity, suggesting the possible usefulness of this and other probiotics in protection against aflatoxicosis (El-Nezami *et al.*, 2002; Gratz *et al.*, 2007). Various non-nutritive sequestering agents, such as polymers, chlorophyll, and yeast-derived products, and silicate minerals and activated charcoal products minimize the toxicological effects of mycotoxins and reduce the potential carryover into the human food chain. They have been reviewed for their potential as additives in livestock feed and make for further in-depth reading (Diaz, 2008).

## Fusariotoxins

Fungi belonging to *Fusarium* spp. and several other genera produce chemically distinct mycotoxins in feed grains and nuts. Type A and type B trichothecenes, zearealenone (ZEN), and fumonisins are fusariotoxins of relatively greater importance from a poultry production and health perspective (Girgis and Smith, 2010). Type A trichothecenes include T-2 toxin, HT-2 toxin, and diacetoxyscirpenol; type B trichothecenes include deoxynivalenol (DON, vomitoxin), 3- and 15-acetyl-DON, nivalenol, and fusarenon-X (D'Mello *et al.*, 1997). Of these, DON is the most commonly encountered fusariotoxin; 15-acetyl-DON is more prevalent in North America, often at levels of 10–20% of DON compared to 3-acetyl-DON prevalent in Europe (Scientific Committee on Food and European Commission, 1999). 15-Acetyl-DON is considered less toxic than DON, whereas 3-acetyl-DON is similarly toxic to DON (U.S. Wheat and Barley Scab Initiative, 2007). ZEN commonly co-occurs with trichothecenes (Placinta *et al.*, 1999). A descending order of sensitivity to fusariotoxins has been noted in turkeys, layers, breeders, broilers, and ducks (Girgis and Smith, 2010). Fusariotoxins differ from other mycotoxins in that they tend to be produced during the colder seasons of the year. Common substrates involved in fusariotoxin production include corn, wheat, barley, oats, peanuts, and, sometimes, forages (Friend and Franson, 1999). The effect of multiple fusariotoxins (DON, 15-acetyl-DON, ZEN, and fusaric acid) in blends of naturally contaminated wheat and corn was evaluated in broiler breeder chicken and other species. Reduced egg production and eggshell thickness accompanied with a significant increase in early embryonic mortality was observed, although the fertility of roosters was not affected (Smith *et al.*, 2008). More studies are needed

to better understand toxicological interactions of fusariotoxins under field conditions. ZEN often occurs with DON in naturally contaminated cereals, and it is responsible for reproductive disorders due to its estrogenic effect at suitable concentrations in susceptible livestock and avian populations. However, in general, ZEA has limited toxicity to birds. Commonly observed symptoms are vent enlargement and enhanced secondary sex characteristics. ZEN may be an indicator of the presence of other mycotoxins and therefore may be of some value as a diagnostic marker. Identification and measurement of suspect mycotoxins are crucial to accomplish a confirmatory diagnosis.

## T-2 toxin

Compared to DON, T-2 toxin occurs less frequently in grain and other agricultural products (Murphy *et al.*, 2006). Weather conditions, grain defects, and moisture contents (13–22%) influence T-2 production, with maximum production at temperatures below 15°C (Mateo *et al.*, 2002). In 7-day-old broilers, the LD<sub>50</sub> of T-2 toxin is 4.97 mg/kg, and it is therefore more toxic than aflatoxin (LD<sub>50</sub> = 6.8 mg/kg), HT-2 toxin (LD<sub>50</sub> = 7.22 mg/kg), and DON (LD<sub>50</sub> = 140 mg/kg) (Leeson *et al.*, 1995). In poultry, toxicological and immunomodulatory effects of T-2 are observed on the liver and digestive system, nervous system, and skin due to its cytotoxic and genotoxic potential, leading to impairment of performance. Type A trichothecenes (especially T-2) are significantly less immunotoxic than type B trichothecenes (Sharma, 1993). However, T-2 toxin can induce necrosis and depletion of lymphoid cells in the thymus, spleen, and lymph nodes of chicken and pullets (Wyatt *et al.*, 1973; Hoerr, 2003). In the digestive system, necrotic damage characterized by white-yellowish bulges containing caseous-necrotic material is observed in various areas of the mouth, gizzard, intestinal mucosa, and liver (Wyatt *et al.*, 1972; Ademoyero and Hamilton, 1991; Konjevic *et al.*, 2004). Frequently, long-term feeding with contaminated feed (1–5 mg/kg) for at least 1 week or even a single application (5 mg/kg) of T-2 can lead to oral lesions and decreased weight gain (Brake *et al.*, 2000). Other type-A trichothecenes, such as HT-2 toxin, diacetoxyscirpenol, monoacetoxyscirpenol, and scirpentriol, can also cause similar lesions (Hoerr, 2003).

The primary toxic effects of T-2 are on the liver, by virtue of inhibition of protein synthesis and reduced enzymatic activity leading to lipid peroxidation and cellular damage (Smith, 1992). Incoordination, hysteroid seizures, and loss of appetite are some central nervous system (CNS) symptoms recorded in birds fed trichothecenes-contaminated feed. Consumption of feed containing elevated concentrations of T-2 (4–16 mg/kg) was correlated with very low feather quality and

wing abnormalities (Wyatt *et al.*, 1975). Comb cyanosis, skin depigmentation of the legs, and necrohemorrhagic dermatitis have been recorded as well. Eggshell thinning, reduced egg production, decreased feed intake and weight gain, and growth retardation are commonly observed signs.

Diagnosis is based on history, clinicopathological findings including necrotic lesions in the digestive system, and detection of T-2 at significant concentrations in the suspected feed. Total amount of all trichothecenes including T-2 toxin in poultry feed should preferably not exceed 0.5 mg/kg, based on a compilation of data including occurrence, toxicity, and clinical signs in poultry (Eriksen and Pettersson, 2004).

### Deoxynivalenol (vomitoxin)

Diversion of DON-infected cereals to poultry feed is commonly believed to be a significant route of exposure. DON is the least acutely toxic but a more commonly occurring grain contaminant (Rotter *et al.*, 1996). Most poultry experimental studies have revealed a highly variable effect on performance, and there is major interest in the effect of chronic low-level exposure. Direct effects on hematology and clinicochemical parameters are poorly defined, although reduced feed intake and weight gain have been established.

Dietary concentrations greater than 5 mg/kg are necessary to cause detrimental effects in broilers (Dänicke *et al.*, 2001), and even higher concentrations did not consistently induce ill-effects. Reduced weight gain and feed refusal were observed at 16–20 mg/kg concentrations of DON. The USFDA has established a 10 mg/kg advisory concentration for DON in grain or grain byproducts destined for chicken, with the added recommendation that such feed may not exceed 50% of the total diet. Erosions in the mucosa of the gizzard, corrugations in the gastric mucosa, duodenitis, jejunitis, intestinal bleeding, and necrosis have been associated with DON exposure. Decreased absorption of glucose and amino acids in the chicken small intestine has been recorded as well. A reduced immune response to Newcastle disease vaccine has been measured in chicken fed 50 mg/kg DON (Harvey *et al.*, 1991), and decreased serum antibody titers against infectious bronchitis virus and Newcastle disease virus have been observed in laying hens and broilers due to feeding DON-contaminated grain.

### Fumonisin

As a commonly occurring contaminant of corn and other agricultural products, fumonisins exert their toxic effects in poultry at relatively higher concentrations compared

to those in pigs. Fumonisin in excess of 100 mg/kg in the diet have been shown to reduce poultry performance. FB<sub>1</sub> incorporation at 251 mg/kg in broiler feed caused a 5% reduction in growth rate, whereas pigs showed a similar growth reduction at 21 mg/kg (Dersjant-Li *et al.*, 2003). In day-old broiler chicks fed 0–400 mg/kg FB<sub>1</sub> for 21 days and 30 mg/kg for 14 days, reduction in weight gain, hepatic necrosis, biliary hyperplasia, and thymic cortical atrophy were noted (Brown *et al.*, 1992). Elevated sphinganine (Sa)/sphingosine (So) ratios were observed in young chicks treated with FB<sub>1</sub> containing culture material (Weibking *et al.*, 1993). Abnormal erythrocyte formation and cytotoxic effects on lymphocytes have been noted as well (Dombrink-Kurtzman *et al.*, 1993). FB<sub>1</sub> incorporated at 50 mg/kg in the diet led to reduced feed intake in turkey poults but not in broiler chicks up to market age; Sa/So ratios were elevated in both, although neither hematologic parameters were affected, nor were any microscopic lesions observed in organs (Broomhead *et al.*, 2002). The USFDA guidance concentration for fumonisins in poultry feed is set at 50 ppm in the total ration. Feed containing elevated fumonisin concentrations may be disposed or diluted with suitable feed sources.

### Ochratoxin and citrinin

These nephron- and hepatotoxic mycotoxins are produced by multiple species of *Aspergillus* and *Penicillium*, and they occur in grains and feedstuff produced worldwide. Ochratoxin A (OTA) is comparatively more toxic than citrinin and has been studied extensively (Gupta, 2011), although both may co-occur. OTA inhibits protein biosynthesis, accelerates lipid peroxidation, causes oxidative stress, and reacts with enzymes-utilizing phenylalanine as a substrate, thereby exerting its toxic effects. The nephrotoxic effect of OTA is due to its action on the organic anion transport system, located in basolateral and brush border membranes of the proximal tubule cells of the nephron and also involved in the absorption/reabsorption and excretion of OTA in the kidney. The middle (S2) and terminal (S3) segments of the proximal tubule of isolated nephrons have been found to be most sensitive to the toxic effect of OTA (Ringot and Chango, 2010). Field outbreaks of ochratoxicosis typically result in decreased feed consumption, efficiency, and growth rate (Hamilton *et al.*, 1982), along with increased mortality (Elaroussi *et al.*, 2006). Avian species vary in their sensitivity to OTA; the LD<sub>50</sub> in chicken is reported to be 2–4 mg/kg, whereas ducks and Japanese quail are susceptible to 0.5–16.5 mg/kg body weight (BW) (Peckham *et al.*, 1971). Young broiler chickens were fed four treatments of feed containing 0, 1, 2, and 4 mg OTA/kg of feed and sequentially euthanized and necropsied up to 21 days of age. At 4 mg/kg (ppm) feed concentrations,



significant growth depression occurred, as early as 6 days of age. Dietary OTA significantly increased the relative weights of liver, kidney, spleen, pancreas, and gizzard. Anemia, characterized by a significant decrease in packed cell volume and hemoglobin levels, was present. Hepatotoxicity was inferred through a significant reduction in serum levels of total protein, albumin, globulin, cholesterol, triglyceride, and blood urea nitrogen and a significant increase in the serum activities of  $\gamma$ -glutamyl transferase. A significant increase in serum uric acid and creatinine concentrations was considered to be indicative of nephrotoxicity (Huff *et al.*, 1988). Dietary OTA at or above 400  $\mu\text{g}/\text{kg}$  (ppb) *ad libitum* feed was fed to broiler chickens for 5 weeks, leading to marked degenerative changes in the kidney and bursa, accompanied by mononuclear infiltration in the liver. Significant increases in the weights of kidney and liver were observed, along with increases in serum glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, uric acid, and creatinine concentrations. Serum total protein, albumin, and globulin were significantly decreased (Elaroussi *et al.*, 2008). Microscopically, pathological changes in the kidney consistent with acute, subacute, and chronic ochratoxicosis have been well characterized and presented in several avian pathology texts.

Broiler chicks fed 300  $\text{mg}/\text{kg}$  (ppm) citrinin displayed severe enlargement of the kidneys, pale livers, and pinpoint cardiac hemorrhages. Histologically, severe tubular nephrosis, vacuolar degeneration of hepatocytes, catarrhal enteritis, degeneration, and mononuclear infiltration in the myocardium were observed (Ahmad *et al.*, 2009). Comparisons between the previously mentioned reports illustrate the difference between toxic doses of OTA and citrinin necessary to cause intoxication.

## Zootoxins

There are few reports of bird intoxication resulting from exposure to zootoxins. Snakebite was believed to have been responsible for the deaths of two red-tailed hawks (*Buteo jamaicensis*) and a Cooper's hawk (*Accipiter cooperii*) based on the carcasses being found in the vicinity of venomous snakes and gross findings of hemorrhages, muscle degeneration, and gangrenous necrosis of a limb (Heckel *et al.*, 1994). Chickens, turkeys, and ducks have also reportedly been killed by snakes based on bite sites being identified on postmortem examination along with the occurrence of compatible gross organ lesions (Lawal *et al.*, 1992).

## Bacterial toxins

### *Botulinum toxin*

Avian botulism results from the ingestion of botulinum toxin produced by *Clostridium botulinum*. There are eight

antigenically distinct toxin types, which are designated A, B, C alpha, C beta, D, E, F, and G; the type designations reflect their order of discovery. Type C alpha cultures produce three toxins designated C1–C3 and small amounts of type D. *Clostridium botulinum* is an anaerobic bacterium that persists in the form of dormant spores when environmental conditions are not conducive for bacterial growth. The spores are quite resistant to destruction and can remain viable for years. Spores of type C toxin are widely distributed in wetland sediments and in tissue of wetland organisms. A number of factors play a role in the occurrence of an outbreak of botulism, including optimal environmental conditions for spore germination and bacterial growth, availability of suitable material to support bacterial replication, and a means for toxin transfer to birds (Friend and Franson, 1999). Most outbreaks occur during summer and fall months when ambient temperatures are relatively high. Bacterial replication requires substrates that are high in protein; decomposing carcasses are good bacterial substrates. Types C1 and D are produced by clostridia containing pseudolysogenic *tox+* phages encoding for the respective toxin genes (Zechmeister *et al.*, 2004; Sakaguchi *et al.*, 2005). Genes encoding for C1 toxin are ubiquitous in wetlands inhabited by bird populations (Zechmeister *et al.*, 2004).

Botulinum toxin is one of the most toxic chemicals known, although for any given species, toxicity varies by toxin type and age (Barnes, 2003). For example, chickens, turkeys, pheasants, and peafowl are susceptible to types A, B, C, and E but not to types D or F (Gross and Smith, 1971). As broiler chickens age, they become less susceptible to type C toxin (Barnes, 2003). Most outbreaks of botulism in birds are due to exposure to type C1 toxin. Less frequently, exposure to type E has caused mortality of fish-eating birds (Friend and Franson, 1999). Domestic chickens have been intoxicated by type A. A mosaic neurotoxin (two-thirds type C and one-third type D) has been characterized that is believed to have been responsible for some forms of avian botulism (Takeda *et al.*, 2005).

Avian botulism is one of the most important diseases of migratory birds (Friend and Franson, 1999). Filter feeding and dabbling waterfowl are among the species most at risk. Raptors have been intoxicated by feeding on poultry carcasses that have not been disposed of properly. Waterfowl are exposed to botulinum toxin via ingestion of contaminated substrates or via contaminated invertebrates such as maggots that feed on decaying carcasses or other organisms such as zooplankton that are inadvertently contaminated. As few as two maggots can be lethal if ingested. Outbreaks of botulism in avian wildlife result in losses that are quite variable, ranging from a few hundred birds to more than 1 million.

Poultry and other production birds can be affected. Game farm pheasants have been intoxicated by feeding on maggots (Foreyt and Abinanti, 1980). Wound contamination by spores of *C. botulinum* was hypothesized to have contributed to the deaths of caponized chickens (Trampel *et al.*, 2005).

Vultures and other carrion eaters are resistant to botulinum toxin, perhaps, in part, due to the production of antibodies to botulinum toxin, which does not occur in sensitive species such as chickens and ducks (Gross and Smith, 1971; Ohishi *et al.*, 1979; Friend and Franson, 1999).

Clinical signs are consistent with neuromuscular paralysis. Early, affected waterfowl are unable to sustain flight (Friend and Franson, 1999). Leg paralysis in waterfowl causes birds to propel themselves using their wings. Paralysis of the nictitating membrane and neck muscles follows, resulting in an inability to hold the head erect (thus the term limberneck). In chickens, an ascending flaccid paralysis is noted (Barnes, 2003). Initially, affected birds are reluctant to walk and, when forced, appear lame. Similar to waterfowl, the wings droop and the neck becomes weak, followed by eyelid paralysis. Waterfowl often drown; affected terrestrial birds die from respiratory and cardiac failure. The time between the onset of clinical signs and death can be as short as a few hours. Postmortem lesions are generally not observed; in affected waterfowl, lesions may be consistent with drowning.

A presumptive diagnosis is based on characteristic clinical signs and environmental conditions conducive to bacterial growth and toxin production. Confirmation of botulism is most often made by using the mouse bioassay to detect the presence of the toxin. Toxin specificity is determined by protecting inoculated mice with specific antitoxins. Enzyme-linked immunosorbent assay and liquid chromatography–mass spectrometry methods have been developed to confirm the presence of toxin (Zechmesiter *et al.*, 2004; Barr *et al.*, 2005). The mouse bioassay is still considered to be the “gold standard” assay due to the sensitivity of the mouse to botulinum toxin. However, newer methods will likely replace the mouse bioassay in the future.

Treatment of individual affected birds with antitoxin can result in recovery, especially in waterfowl. Other affected species, such as coots, shorebirds, gulls, and grebes, rarely recover (Friend and Franson, 1999). Difficult logistics, limited availability of antitoxin, and cost of treatment preclude effective intervention in large outbreaks.

Prevention and control of outbreaks are critical to minimize losses. In wetlands, the amount of organic and decaying material should be limited. Prompt removal and disposal via burial or burning of animal carcasses is important during outbreaks to interrupt bacterial growth and toxin production. Immunization with inactivated

bacterin-toxoids is protective, but vaccination of large poultry flocks or waterfowl is expensive and, in the case of waterfowl, logistically difficult.

#### *Biogenic amines*

Biogenic amines, such as gizzerosine, histamine, histidine, cadaverine, spermine, and putrescine, result from the heating or bacterial spoilage of fish and animal byproducts (Barnes *et al.*, 2001; Brown and Julian, 2003). Poultry are potentially exposed to the amines when fish or meat meal is incorporated into their diets. Toxic concentrations vary depending on the specific amine. Dietary histamine at 0.1% is associated with reduced feed conversion and body weights in broiler chickens (Barnes *et al.*, 2001). Dietary spermine is toxic to 120-week-old chicks at 0.2% (Sousadias and Smith, 1995). Putrescine is toxic at concentrations of 0.05% (Chowdhury, 2001). Interestingly, a dietary concentration of putrescine of 0.05% has been associated with improved eggshell quality (Chowdhury, 2001). The improvement was hypothesized to be due to increased calcium transport.

Biogenic amines at toxic concentrations cause digestive disturbances. Gizzerosine causes gizzard erosion and hemorrhage. A dietary histamine concentration of 4ppm results in localized gizzard lesions and reduced growth rates in chicks (Harry and Tucker, 1976). In addition to gastrointestinal effects, toxic biogenic amine concentrations cause a malabsorption syndrome in chickens characterized by reduced feed efficiency and enlargement of the proventriculus (Barnes *et al.*, 2001).

## Feed additives

### *Arsenicals*

Arsenic exists in a variety of inorganic and organic forms and valences. Arsenic trioxide was used as an herbicide. Pentavalent and trivalent sodium, potassium, and calcium salts of arsenic have been used as insecticides, especially for ants. Trivalent organic forms of arsenic include the herbicides monosodium methanarsonate and disodium methanarsonate and thiacetarsamide, previously used for treating dogs with heartworm. Pentavalent arsenicals used as feed additives include arsanilic acid, sodium arsanilate, 3-nitro-4-hydroxyphenylarsonic acid (3-nitro and roxarsone), and 4-nitrophenylarsonic acid. Arsanilic acid and 3-nitro are the most commonly used organic arsenicals in poultry production (Feed Additive Compendium, 2005). They are used to increase feed efficiency, promote growth, improve pigmentation, and prevent coccidiosis. Although exposure of birds to inorganic arsenic salts can occur, most avian arsenic intoxications involve exposure of poultry to pentavalent organic arsenicals.

Pentavalent organic arsenicals are believed to substitute for phosphate in oxidative phosphorylation. Uncoupling of oxidative phosphorylation results in cellular energy deficits (Ensley, 2004). Intoxication occurs most commonly due to feed mixing errors. Clinical signs have occurred in turkeys exposed to dietary concentrations of 3-nitro approximately twice the recommended dose. Clinical signs in affected birds include stunting, depression, apparent lameness, and ataxia. Gross lesions are typically absent, but histopathologically, Wallerian degeneration of the optic and peripheral nerves occurs. Wallerian degeneration can also occur in the spinal cord (Ensley, 2004). Ulcerative cholecystitis has been reported in turkey poultlets intoxicated by 3-nitro (Brown *et al.*, 1991).

Diagnosis of organic arsenical intoxication in poultry depends on detection of potentially toxic concentrations in representative feed samples and the occurrence of compatible clinical signs and postmortem lesions. Treatment consists of removing the offending feed and providing accessible feed and water.

Arsenic has been detected at relatively high concentrations in some seabirds, including albatrosses and gulls (Kubota *et al.*, 2003). It is present in tissues primarily as the organic arsenical, arsenobetaine, which is relatively nontoxic.

A significant percentage of the human tolerable daily intake of arsenic can come from arsenic residues in poultry meat (Taylor, 2004). Measurable increases in soil arsenic concentrations can occur as a result of using litter from arsenic-treated poultry as fertilizer (Garbarino *et al.*, 2003; Rutherford *et al.*, 2003).

## Sodium

Sodium ion intoxication is a significant problem in poultry production. Intoxication can result from excessive sodium in the feed, water deprivation, or ingestion of saline waters (Brown and Julian, 2003). The most common form of sodium associated with intoxication is sodium chloride. Sodium sesquicarbonate, used as a buffering agent in ruminant feeds containing high concentrations of urea, was responsible for high mortality in broiler chickens as a result of a feed misformulation (Sander *et al.*, 1998). High dietary sodium can be well tolerated if access to water is not restricted. For example, 15-day-old turkey poultlets tolerated up to 10% sodium chloride in their mash for 14–16 days when drinking water was not restricted (Gitter *et al.*, 1979). However, young chicks and poultlets can be intoxicated from sodium in feed despite adequate water intake. Sodium concentrations in both the feed and the water need to be considered in assessing sodium exposure.

A number of factors influence the toxicity of the sodium ion, including bird age, bird species, dietary factors, and water quality and accessibility. Young birds are

more sensitive to sodium ion toxicity, most likely due to less developed renal function. Turkeys are more susceptible to intoxication than are chickens (Berger, 1993). In addition, birds have less ability to excrete salts in excess of water, so their ability to reduce high-plasma osmolality is limited (Barnes, 2003). Some waterfowl have nasal salt glands that provide an additional excretory route. In general, the addition of 1% salt (as sodium chloride) to poultry rations is safe, even for very young birds. A level of 0.25% salt in drinking water is considered to be safe (Berger, 1993).

At high sodium intakes, birds develop acute, severe diarrhea and dehydration, lose weight, and die. Renal function is often impaired, especially if sodium bicarbonate is the source for exposure (Barnes, 2003). Lower, but still toxic, sodium intakes primarily cause cardiac overload, ascites, dyspnea, and edema. Loose droppings, decreased feed intake, and poor growth are often observed. Thirteen-week-old tom turkeys affected by high dietary sodium chloride concentrations (~8%) exhibited polydipsia, diarrhea, ataxia, tremors, depression, sternal and lateral recumbency, torticollis, and death (Wages *et al.*, 1995).

Ascites, edema, fluid in the lungs, hydropericardium, cardiac hypertrophy (right-sided in chickens and bilateral in poultlets), and dilatory cardiomyopathy (poultlets) are found on postmortem examination. Most microscopic lesions are secondary to cardiac failure, although bilaterally symmetrical areas of cerebral necrosis, vascular congestion, and edema were noted in intoxicated tom turkeys (Wages *et al.*, 1995).

Sodium intoxication in waterfowl has been associated with the ingestion of hypersaline water or salt that has precipitated on feathers (Windingstad *et al.*, 1987; Meteyer *et al.*, 1997; Gordus *et al.*, 2002; Stolley and Meteyer, 2004). Significant gross and microscopic lesions in ruddy ducks intoxicated by sodium include conjunctivitis; lens opacity; cataract formation; vascular congestion in multiple organs, especially in the meninges; and myocardial and skeletal muscle degeneration (Gordus *et al.*, 2002).

The use of sodium chloride as a deicing agent on roads is believed to intoxicate passerine birds. Experimentally, sodium chloride was lethal to house sparrows dosed with a concentrated sodium chloride solution providing 8000 mg/kg (0.8%) (Bollinger *et al.*, 2005).

A diagnosis of sodium ion intoxication relies on detection of high dietary and/or water, serum, and/or brain sodium concentrations. The significance of detected dietary and water sodium concentrations needs to be assessed in conjunction with other historical, clinical, and postmortem findings. Serum sodium concentrations greater than 150 mEq/L are elevated, and brain sodium concentrations greater than 2000 ppm wet weight are highly suggestive of intoxication (Puls, 1994).

### Vitamin A

Vitamin A is an essential micronutrient that plays a role in normal vision, reproduction, immunity, membrane integrity, growth, and embryogenesis. Vitamin A intoxication has been produced experimentally in poultry and cockatiels (Tang *et al.*, 1985; Koutsos *et al.*, 2003). Osteodystrophy occurred in broiler and leghorn chicks exposed to 330–660 IU vitamin A per day for 21 days (Tang *et al.*, 1985). Skeletal development was affected in growing chickens given 200 mg retinyl acetate per kilogram (Baker *et al.*, 1967). Anorexia, conjunctivitis, eyelid adhesions, and encrustations around the mouth were also noted by Tang *et al.*, 1985. Cockatiels fed approximately 30,000 µg/kg of vitamin A (as retinol) for 269 days exhibited intensified vocalization patterns, pancreatitis, multifocal accumulation of lymphocytes in the lamina propria of the duodenum, and reduced body condition (Koutsos *et al.*, 2003). Intakes of 3000 µg/kg of vitamin A also caused clinical signs, but they were less severe. A diagnosis of vitamin A intoxication can be made based on measurement of high vitamin A concentrations in representative feed samples and in serum or liver tissue along with the occurrence of compatible clinical signs.

### Vitamin D

Vitamin D is required for normal calcium and phosphorus homeostasis, which in turn is important for normal bone, beak, claw, and eggshell formation. Poultry diets are commonly supplemented with vitamin D in the form of cholecalciferol (D<sub>3</sub>) (Barnes, 2003). Vitamin D stimulates the absorption of calcium from the GI tract, influences osteoblast and osteoclast activity, and increases renal tubular reabsorption of calcium. Exposure to excessive vitamin D results in increases in calcium absorption from the GI tract and renal tubules and also calcium mobilization from bone. High blood and tissue calcium concentrations cause metastatic tissue calcification. In broiler chicks, dietary concentrations of 30,000 IU/kg are toxic when fed during growth. Much higher doses of vitamin D can rapidly cause renal calcification and damage. Aortic and arterial calcification can also occur. Postmortem lesions include parathyroid gland atrophy, renal tubular and aortic calcification, and calcification of blood vessel walls in the brain. Older birds are less sensitive than younger, actively growing birds to vitamin D. Other forms of vitamin D are also potentially toxic. Relative toxicity is vitamin D<sub>2</sub> < vitamin D<sub>3</sub> < 25-mono-hydroxycholecalciferol < 1,25-dihydroxycholecalciferol (Barnes, 2003). Nephrocalcinosis was experimentally produced in chicks by feeding powdered leaves from *Cestrum diurnum*, which contains an analog of 1,25-dihydroxycholecalciferol (Sarkar *et al.*, 1981).

### Ionophores

Monensin, lasalocid, salinomycin, narasin, maduramicin, and semduramicin are the major, approved ionophore antibiotics used in poultry. Accidental or intentional off-label use has resulted in adverse reactions in adult poultry (laying hens), ostriches, and ornamental or game birds. In addition, interactions with other drugs used in target and non-target species can occur (Dorne *et al.*, 2011).

Salinomycin toxicity in turkeys has been reported extensively. A case report describing the death of 4287 (34.5%) 19-week-old heavy hybrid turkey hens in a flock in Manitoba ascribed the mortality to the accidental feeding of a broiler premix containing salinomycin sodium at 60 ppm concentrations along with 50 ppm of bacitracin methylene disalicylate. Affected birds showed signs of dyspnea, drowsiness, sternal recumbency with legs extended posteriorly, inability to stand, stiffness, and weakness. Histological muscle sections revealed extensive fragmentation and necrosis of muscle fibers. Myocardial fibers were eosinophilic and undergoing fragmentation. No other lesions were found. Salinomycin toxicity increases with age in turkeys. In one study, when turkeys at 27 or 32 weeks of age were fed diets containing 44–66 ppm salinomycin, 13 of 20 birds died. In contrast, 7-week-old turkeys fed the same concentrations showed a mortality of only 1 of 84 (Potter *et al.*, 1986).

Another case report of monensin intoxication on a commercial ostrich farm in northern Greece described clinical signs in 7 adult birds from a flock of 24 African black breeding ostriches. A locally manufactured vitamin and mineral supplement was used to replace a previously used premix, leading to the development of clinical signs of depressed appetite, sternal recumbency, dehydration, dyspnea, and diarrhea. Monensin was detected at 3790 ppm in the supplement and at 190 ppm in the feed concentrate (Dedoussi *et al.*, 2007). Extremely elevated concentrations of serum aspartate aminotransferase, creatine kinase, and lactate dehydrogenase were suggested to be caused by acute muscle myopathy and, in addition to the clinical signs and age of birds, were utilized to differentiate the toxicosis from botulism, salt poisoning, mycotoxicoses, vitamin E–selenium deficiency, and toxic plant ingestion. There are no indications for the therapeutic use of ionophores in ostriches, and no data are available on the safe levels of inclusion of these drugs in ostrich feed.

Various antibiotics have been reported to potentiate ionophore toxicity (Novilla, 2007). The most frequently reported drug interaction is with the pleuromotilin derivative, tiamulin. This antibiotic interferes with the metabolic degradation of monensin in the liver, causing accumulation at toxic concentrations. In another study, concurrent administration of tiamulin with semduramicin was evaluated in grower broilers. A temporary growth depression and transiently reduced feed efficiency was observed during the third week of the trial in the group receiving both antibiotics



simultaneously. However, by the fourth and the fifth (final) week, no adverse effects were observed and the feed conversion was found to be improved. Histopathological and hematological parameters were unaffected at the end of the trial (Schuhmacher *et al.*, 2006).

## Drugs

A number of chemotherapeutic agents have caused intoxications in birds, primarily in poultry and pet birds. In most cases, intoxication results from inappropriate

or overuse of a drug. Table 67.2 lists chemotherapeutic agents associated with adverse effects in birds. Two drugs have been documented to intoxicate birds of prey as a result of feeding on carcasses from animals administered the drugs prior to death. Carcass residues of the nonsteroidal anti-inflammatory diclofenac have been shown to cause renal failure, visceral gout, and death in oriental white-backed vultures (*Gyps bengalensis*) and long-billed vultures (*G. indicus*) in the Indian subcontinent (Oaks *et al.*, 2004; Shultz *et al.*, 2004). Diclofenac has been a widely used veterinary drug in regions with affected birds. In the United States, use of pentobarbital for euthanasia

TABLE 67.2 Drug intoxications in birds

Drug	Effects	Clinical signs	Pathologic lesions	References
Sulfonamides	Blood dyscrasias, bone marrow depression, anemia, thrombocytopenia, lymphoid depression, impaired immune function	Depression, pallor, weight loss, decreased egg production and quality, icterus, depigmentation of brown eggs	Widespread hemorrhage; pale bone marrow; swollen, pale red, or icteric liver; splenic enlargement; ulcers at proventricular–gizzard junction	Reece <i>et al.</i> (1985), Daft <i>et al.</i> (1989), Brown and Julian (2003)
Nitrofurans	Furazolidone causes dose-related biventricular cardiomyopathy; chronic exposure can delay sexual maturity in male broiler breeder chickens	Depression, incoordination, ruffled feathers, growth retardation, hyperexcitability, tremors, loud vocalization, opisthotonus, aimless running, seizures	Dilation of ventricles of the heart, thinning of right or left ventricular wall, passive congestion with lung edema, liver congestion, ascites	Reece <i>et al.</i> (1985), Brown and Julian (2003)
Aminoglycosides	Lysosomal dysfunction in renal tubular epithelial cells leading to necrosis; impaired synthesis of protective vasodilatory renal prostaglandins	Gentamicin causes depression, edema, injection site hemorrhages, large pale nephritic kidneys, weakness, and apnea	Increased number of lysosomes containing myelin figures in renal tubule cells	Bird <i>et al.</i> (1983), Boothe (2001), Woolley <i>et al.</i> (2001), Brown and Julian (2003)
	Apoptosis in cochlear sensory hair cells	Ototoxicity Egg inoculation associated with embryo deaths	Renal tubular cell necrosis Degeneration of the apical portion of cochlear hair cells	
		Streptomycin and dihydrostreptomycin sulfate associated with respiratory distress, paresis, mild convulsions		
Nicarbazin	Increases metabolic rate and heat production; increases lipoprotein lipase activity; acts as a Ca <sup>2+</sup> ionophore	Poor eggshell pigmentation, decreased egg weight, decreased egg hatchability, growth depression, increased susceptibility to heat stress	No diagnostic lesions	Reece <i>et al.</i> (1985), Hughes <i>et al.</i> (1991), Brown and Julian (2003), Yoder <i>et al.</i> (2006)
Dimetridazole (other nitroimidazoles)	Possible free radical damage to DNA and other molecules	Growth depression, decreased egg production, incoordination, inability to fly, aimless running, vocalization, tremors, seizures	No diagnostic lesions	Riddell (1984), Brown and Julian (2003)
3,5-Dinitro- <i>o</i> -toluamide	Purkinje cell dysfunction	Ataxia, torticollis, reduced growth	Necrosis and depletion of Purkinje cells of the cerebellar cortex	Reece and Hooper (1984), Brown and Julian (2003)
Ivermectin (and other macrolide endectocides)	GABA agonist	Somnolence, listlessness, ataxia, bradypnea, mydriasis	No diagnostic lesions	Kim and Crichlow (1995), Brown and Julian (2003)

has resulted in secondary intoxication of raptors feeding on carcasses from euthanized animals (O'Rourke, 2002). Intoxication has occurred from scavenging carcasses not disposed of properly on farms or landfills.

## Pesticides

A tabulation of the toxicity of a number of pesticides for birds is provided in Table 67.3.

### Insecticides

#### *Organochlorine insecticides*

Historically, OCs were widely used from the 1940s to the 1970s in agriculture and forestry and for mosquito control. The most widely used organochlorines included the dichlorodiphenylethanes (DDT, methoxychlor, and dieldrin), cyclodienes (aldrin, dieldrin, heptachlor, chlordane, and endosulfan), and hexachlorocyclohexanes (lindane and benzene hexachloride). Although these insecticides are no longer used in the United States, Canada, and Europe, they continue to be used in developing countries. These compounds are highly lipophilic and, in general, have long environmental and body half-lives. Because of their lipophilic and persistent nature, they biomagnify within food webs. They were banned due to their environmental persistence, concern about their impact on human health and wildlife health, and widespread insect resistance.

#### *Cholinesterase inhibitors (organophosphorus and carbamate insecticides)*

Due to their more rapid breakdown in the environment, OP and carbamate insecticides replaced the OC pesticides as the latter were banned for use in North America and Europe in the 1960s and 1970s. A number of different formulations are available for use either in the environment (e.g., agricultural or residential use) or on animals (e.g., livestock dips or sprays). OPs and carbamates are formulated as liquids, granules, and powders. The more toxic insecticides of each group are generally restricted to agricultural uses, whereas less toxic members are approved for use on animals or in residential environments. Although chemically distinct, the OPs and carbamates have a common mechanism of toxic action, namely the inhibition of cholinesterase enzymes.

Possible exposure scenarios are numerous. Pet birds can be exposed via their diets, via home or premise use, or via direct application. Inhalation exposure is also possible from the use of dichlorvos-impregnated pest strips or premise spraying or fogging. Poultry and other production birds can be exposed in similar ways. There are relatively few documented reports of acute pet or

production bird intoxication from the use of OPs or carbamates. However, acute OP and carbamate intoxications are common in wild birds, and although the number of affected birds is difficult to assess, losses are significant. For example, at the height of use of granular formulations of the carbamate insecticide carbofuran, 17–91 million birds were estimated to have died annually (Mineau, 2005).

Exposure of wild birds to these insecticides can occur via ingestion of treated seeds or vegetation (accidental, intentional, or misuse of a product), poisoned insects or animals (impaired live animals or carcasses), product (especially granular formulations), or contaminated water (Friend and Franson, 1999). Inhalation or dermal exposure is also possible from spraying or spills. With avian wildlife, there can be some degree of seasonality to OP or carbamate intoxications as a result of season patterns of insecticide use.

The acute oral toxicity of individual OPs and carbamates varies considerably within each of their classes. For example, within the carbamate insecticide class, the acute oral LD<sub>50</sub>s for carbofuran and carbaryl in mallard ducks are 0.5 and 0.2 mg/kg BW, respectively (Friend and Franson, 1999). Also, acute oral toxicities of specific OP or carbamate insecticides vary considerably between species. The acute oral LD<sub>50</sub>s of ethion range from 45 mg/kg for blackbirds to 0.2 mg/kg for mallard ducks. A number of other factors can influence toxicity, including age, sex, diet, body condition, and product formulation.

Some OPs, including insecticides such as leptophos, mipafox, and cyanofenphos and industrial chemicals such as tri-*ortho*-cresyl-phosphate, cause a delayed neurotoxic effect referred to as OP-induced delayed neuropathy (OPIDN). OPIDN occurs as a result of inhibition of neurotoxic esterase, an enzyme found in peripheral nerves. OP insecticides are tested for their ability to cause OPIDN in adult hens as a result of their unique sensitivity to this effect. Also, pheasants and mallard ducklings are highly susceptible to delayed neurotoxicity (Brown and Julian, 2003). Most OPIDN-inducing insecticides are no longer on the market (Hill, 2003).

As previously mentioned, OPs and carbamates inhibit cholinesterase enzymes. Inhibition of acetylcholinesterase (AChE) is primarily responsible for the clinical signs associated with intoxication. Enzyme inhibition prevents the breakdown of ACh at the synapses and neuromuscular junctions. As a result, ACh overstimulates muscarinic and nicotinic receptors in the central and peripheral nervous systems. Clinical signs include convulsions, hyperexcitability, opisthotonus, lethargy, miosis or mydriasis, apparent blindness, ataxia, muscular weakness, tachypnea, dyspnea, emesis, defecation/diarrhea, piloerection, lacrimation, ptosis, and epistaxis (Friend and Franson, 1999). The onset of clinical signs and death

TABLE 67.3 Rodenticides, avicides, and molluscicides toxic for avian species

Rodenticides/ avicides/ molluscicides	Uses	Formulations	Avian toxicity	Mechanism of toxic action	Clinical signs	Species affected	References
4-Aminopyridine (Avitrol)	Control red-winged blackbirds, blackbirds, grackles, pigeons, sparrows	Powder concentrate; 0.5–3.0% grain baits; 3% cracked corn bait for agricultural use diluted to 0.03% with untreated grain; 1% whole corn diluted to 0.1% for crows; 0.5% bait for pigeons	Highly toxic for most species, including birds; LD <sub>50</sub> s for birds range from 1.4 to 8.1 mg/kg	Blocks potassium ion channels and increases release of ACh	Distress cries, aerial distress displays, seizures, coma	Potentially all birds	Bischoff <i>et al.</i> (2001), Schell (2004a,b)
Bromethalin	Rodenticide	0.01% tan or green grain- based pellets in 16- to 42.5-g place packs	Not determined	Uncouples oxidative phosphorylation	Not described in birds; clinical signs in mammals dependent on ingested dose, with CNS excitation noted with high doses and paralysis noted with lower doses	Potentially all birds	Dorman (2004)
3-Chloro- <i>p</i> -toluidine hydrochloride (Starlicide)	Control ravens, starlings, crows, pigeons, cowbirds, grackles, magpies, certain gull species	98% powder used to prepare baits; 0.1% ready-to-use product	Highly toxic for most species including birds; LD <sub>50</sub> s for birds of 1.8–3.8 mg/kg BW	Unknown	Decreased activity, tachypnea, dyspnea, renal failure	Potentially all birds	Schell (2004a,b)
Fluoroacetate (1080)	Rodenticide, coyote control	Use restricted to livestock protection collars (LPCs); LPCs for sheep and goats contain 30 mL at 1%	Oral LD <sub>50</sub> s for magpies are 1.78–2.3 mg/kg BW	Blocks tricarboxylic acid cycle resulting in cell energy depletion	Vary depending on species; not well described in birds; CNS, gastrointestinal, and cardiovascular signs likely; skeletal muscle necrosis reported in mallard ducks	Potentially all birds; proper use of LPCs appears to present little or no intoxication risk to avian scavengers	Burns and Connolly (1995), Ataria <i>et al.</i> (2000), Parton (2004)
Metaldehyde	Control slugs and snails	Pelleted baits, granules, liquids, or wettable powders containing 5% metaldehyde	Minimum lethal doses for chickens and ducks are 500 and 300 mg/kg BW, respectively	Decreases GABA in the brain; alterations of other brain neurotransmitters	Restlessness, anxiety, tachypnea, seizures, hyperthermia	Potentially all birds; secondary poisoning not reported	Talcott (2004a,b)
Strychnine	Controlling ground squirrels, meadow and deer mice, prairie dogs, porcupines, chipmunks, rabbits, pigeons	Colored grain-based baits with 0.5–1.0% strychnine sulfate	Reported oral LD <sub>50</sub> s for golden eagles, sage grouse, and pheasants are 5–10, 42.5, and 8.5–24.7 mg/kg, respectively	Blocks inhibitory actions of glycine of spinal cord anterior horn cells and inhibits neurotransmitter release from Renshaw cells	Nervousness, apprehension, anxiety, tachypnea, muscle spasms, stiffness, tonic extensor muscle contractions, death due to respiratory impairment Secondary toxicity reported	Potentially all birds	Redig <i>et al.</i> (1982), Wobeser and Blakley, (1987), Warnock and Schwarzbach (1995), Talcott (2004a,b)
Zinc phosphide	Used to control rats, mice, voles, ground squirrels, prairie dogs, nutrias, muskrats, rabbits, opossums, gophers Aluminum phosphide used as a fumigant	Grain-based bait, scrap bait, paste, or tracking powder; baits generally contain 0.5–2.0% zinc phosphide; paste up to 10%	Oral LD <sub>50</sub> s for wild birds and ducks reported to be 23.7– 37.5 mg/kg; oral LD <sub>50</sub> in chickens reported to be 25 mg/kg	Phosphene released from zinc or aluminum phosphide blocks cytochrome oxidase	Often found dead; dullness, tachypnea, dyspnea, tremors, paralysis, seizures, death  Secondary toxicity unlikely	Potentially all birds	Shivanandappa <i>et al.</i> (1979), Albretson (2004), Poppenga <i>et al.</i> (2005), Tiwary <i>et al.</i> (2005)

can be rapid; birds are often found dead. Death is most often due to respiratory failure and hypoxia. Gross and histopathologic lesions in dead birds are usually minimal and nonspecific. Gastrointestinal contents should be examined carefully because the presence of granules or dye may suggest pesticide exposure. Often, given the rapidity of death, there is freshly ingested food in the upper GI tract. OP inhibition of AChE activity can be irreversible, thus necessitating synthesis of new enzyme before recovery can occur. Carbamates do not irreversibly inhibit AChE, and spontaneous regeneration of enzyme activity is rapid. Clinical signs can persist for days in OP intoxicated animals, whereas recovery from carbamate intoxication is generally completed within 2 or 3 days.

A diagnosis of intoxication is dependent on measuring reduced activity of cholinesterase along with identification of a specific insecticide in suitable antemortem or postmortem samples. In birds, plasma is suitable for cholinesterase activity determinations. Brain cholinesterase activity can be measured in dead birds. Cortex is the preferred specimen for cholinesterase analysis. It is important to note that because carbamate inhibition of cholinesterase activity is readily reversible, even after death, care must be taken when interpreting laboratory results. In general, cholinesterase activity less than 50% of normal suggests significant exposure to an OP or carbamate (activity is often <20% of normal following lethal exposures) (Hill, 2003). Normal plasma/whole blood and brain cholinesterase activities are quite variable among bird species, and interpretation of cholinesterase activity should be based on species-specific and, when possible, laboratory-specific reference ranges. Reference ranges for cholinesterase activity in plasma and brain from a variety of bird species have been published (Westlake *et al.*, 1983; Hill, 1988). Gastrointestinal contents and liver samples should be submitted for detection of a specific insecticide to confirm exposure. Urine should be submitted for detection of major metabolites of OPs (Jain, 2006).

Otherwise sublethal exposure to OPs or carbamates can result in mortality as a result of a variety of effects: increased vulnerability to trauma or predation, reduced ability to regulate body temperature and therefore increased susceptibility to hypothermia, and reduced activity leading to decreased feeding and weight loss (Friend and Franson, 1999; Hill, 2003). In addition, birds can be indirectly affected by reduced availability of food as a result of prey die-offs.

Fortunately, the overall adverse impact of agricultural pesticide use in general, and OPs and carbamate use more specifically, on avian wildlife has lessened during approximately the past decade. This is due primarily to the replacement of older, more toxic insecticides with

newer, less toxic ones such as pyrethrins/pyrethroids and neonicotinoids (Mineau and Whiteside, 2006).

#### *Pyrethrins/pyrethroids*

Naturally occurring pyrethrin (derived from chrysanthemum flowers) and synthetic pyrethroid insecticides are currently estimated to make up more than 25% of insecticide use worldwide. Their popularity is due to their lack of environmental persistence and relatively low toxicity for birds and mammals (they are highly toxic for fish). They are used to control a variety of agricultural, home, and animal pests and are available in a large number of formulations, including sprays, dusts, dips, shampoos, spot-ons, foggers, ear tags, wettable powders, granules, soluble powders, and emulsifiable concentrates. They are often combined with synergists such as piperonyl butoxide to enhance their insecticidal activity.

Pyrethrins and pyrethroids have low toxicity for birds. For example, the acute oral LD<sub>50</sub> of cypermethrin for mallard ducks is greater than 4640 mg/kg, and the dietary LC<sub>50</sub> for mallard ducks and bobwhite quail is greater than 20,000 ppm (EXTOXNET; <http://extoxnet.orst.edu>). The 8-day LC<sub>50</sub> of deltamethrin for ducks and quail is greater than 4650 and greater than 10,000 mg/kg, respectively. Permethrin is practically nontoxic to birds, with acute oral LD<sub>50</sub>s for one permethrin formulation (Praxem) of greater than 9900, greater than 13,500, and greater than 15,500 mg/kg for mallard ducks, pheasants, and Japanese quail, respectively (EXTOXNET).

Pyrethrins and pyrethroids are neurotoxic as a result of their ability to alter sodium channels and cause repetitive nervous discharges or membrane depolarization. Clinical signs associated with acute intoxication are related to nervous system stimulation. There does not appear to be any adverse behavioral effects on avian wildlife following sublethal exposures to pyrethrins/pyrethroids, similar to those reported for OPs or carbamates, which would cause mortality due to increased predation or inability to obtain food. However, this possibility should be further investigated.

#### *Boric acid*

Boric acid is used in poultry litter to control darkling beetles (Brown and Julian, 2003). Recommended litter treatment concentrations are between 0.4 and 0.9 kg per 9.3 m<sup>2</sup> (Dufour *et al.*, 1992). Boric acid has a relatively high acute oral LD<sub>50</sub> for 1-day-old chickens of 2.95 g/kg BW (Sander *et al.*, 1991). Exposure of 1-day-old chicks to litter treated with up to 7.2 kg boric acid per 9.3 m<sup>2</sup> of litter for 15 days exhibited a dose-related feathering abnormality, but no effect on productivity and no lesions were noted. Exposure of poultry to litter treated with recommended amounts of boric acid is unlikely to be toxic.



### Nicotine

Nicotine sulfate has been used to paint chicken roosts to control mites and has been given orally for internal parasites (Brown and Julian, 2003). Nicotine is a highly toxic alkaloid that stimulates nicotinic receptors at neuromuscular junctions. Initial receptor stimulation is followed by a depolarizing blockade. Death is due to hypoxia as a result of paralysis of the diaphragm and chest muscles. Currently, nicotine use as an insecticide is not common. Perhaps of more concern is the passive exposure of pet birds to cigarette, cigar, or pipe smoke. Chronic ocular, dermatologic, and respiratory diseases in pet birds have been associated with tobacco smoke exposure (Dumonceaux and Harrison, 1999).

Neonicotinoids, a new class of insecticide, are also nicotinic receptor agonists. They are considered to have low toxicity for vertebrates because of relatively low affinity for vertebrate nicotinic receptors compared to insect nicotinic receptors (Tomizawa and Casida, 2005, 2011). Neonicotinoids include acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam.

### Rodenticides

#### Anticoagulants

Anticoagulant rodenticides are the most widely available and used rodenticides. Available avian toxicity information is most often expressed as a median lethal concentration (LC<sub>50</sub>) of anticoagulant included in feed over a defined period of time (Petterino and Paolo, 2001). There are few toxicity data available for species such as raptors that are likely to be exposed to the anticoagulants following ingestion of exposed and/or intoxicated prey.

There are almost no reported cases of anticoagulant rodenticide intoxication in poultry. There is one case report of coumatyral toxicity in chickens 1 week of age (Munger *et al.*, 1993). Exposure occurred as a result of contaminated wood-straw mats used to ship the birds. The low incidence of intoxication most likely reflects the relatively controlled environments associated with most commercial poultry production. Exposure is more likely in free-roaming poultry.

There are several documented cases of anticoagulant intoxication in captive wild birds, including waterfowl, raptors, and bird-of-paradise. Fortunately, most institutions housing wild birds recognize risks associated with anticoagulant rodenticide use and either use alternative rodenticides or take precautions to avoid exposure.

Exposure of wild birds to anticoagulants either via bait ingestion or through ingestion of exposed and/or intoxicated prey is a major concern. The long half-lives of second-generation anticoagulants result in intoxication risks associated with repeated exposures to relatively low

doses of the compounds. Avian species that feed primarily on small rodents are at greatest risk. Soon after the introduction of second-generation anticoagulants, the potential for secondary intoxication of raptor species such as owls was investigated (Mendenhall and Pank, 1980; Townsend *et al.*, 1981). Residues have been measured in a number of bird species. Although anticoagulant intoxication has been documented in a number of individual birds, population impacts are less clear.

Diagnosis of intoxication is dependent on antemortem and/or postmortem evidence of a coagulopathy and the detection of an anticoagulant in blood, serum, or liver samples. Merely finding a residue of an anticoagulant in an animal is not sufficient for a diagnosis of intoxication because residues are often found in the absence of a coagulopathy.

Vitamin K<sub>1</sub> is antidotal treatment, although there is some delay before coagulopathy resolves. Dosing regimens have not been well defined in avian species, but vitamin K<sub>1</sub> at 0.2–2.2 mg/kg given intramuscularly or subcutaneously every 4–8 h until the bird is stabilized has been recommended. Once stabilized, vitamin K<sub>1</sub> given at similar doses once daily for 2 weeks or longer is indicated.

#### Avicides

Avicides are marketed primarily to control pest bird species such as blackbirds, pigeons, and grackles. They are designed to be placed on grain for baiting in such a way as to only affect a few individuals in a flock. The clinical signs exhibited by affected birds (distress cries and aerial distress displays) are such that unaffected birds are scared away. Two commonly used avicides are 4-aminopyridine and 3-chloro-*p*-toluidine hydrochloride. A number of chemicals have been investigated for their ability to repel birds (Clark, 1998; Dolbeer *et al.*, 1998; Stevens and Clark, 1998). Repellants such as methyl anthranilate are generally not associated with lethal intoxication.

## METALS

### Lead

Occurrences of lead exposure and intoxication in birds continue to be detected worldwide (Fisher *et al.*, 2006), along with increasing awareness and regulation. These occurrences are commonly due to mining and industrial activities, contaminated waste disposal, paint stripping/flaking, persistent lead-containing ammunition, and various household and hobby-based objects containing significant amounts of lead.

Clinical signs consistent with lead intoxication are related to nervous, GI, hematopoietic, and renal systems. Various species-specific and habitat-linked factors of lead exposure are unique to birds. Often, birds consume lead shot or other lead objects mistaken as seed or grit. The curious nature of pet birds leads them to ingest shiny objects easily. Temporal and spatial patterns of lead intoxication in California condors coinciding with anthropogenic activities such as big game hunting have been recorded (Cade, 2007; Strom *et al.*, 2009).

The amount and form of lead ingested, dietary factors, size of ingested lead particles, and amount of grit in the ventriculus are some factors that influence absorption and subsequent intoxication. In some species of raptors, rapid removal of lead particles can take place by regurgitation from the proventriculus (Locke and Thomas, 1996). Information pertaining to toxic doses is available for a limited number of avian species, although the large number of variables that affect toxicity of lead makes generalizations difficult (Smith *et al.*, 2009).

Inorganic lead salts and organic forms are more bioavailable than elemental lead; however, the acidic environment in the proventriculus and ventriculus increases the solubility of elemental lead. Lead is actively transported across the GI tract through the same transport mechanism used for calcium absorption. Irrespective of its form, ingested lead is mostly excreted in the feces without being absorbed. Red blood cells contain a majority of the absorbed lead, with smaller amounts being bound to albumin or existing as free lead in plasma.

Signs of intoxication can be nonspecific and limited to regurgitation, anorexia, weakness, and weight loss (Puschner and Poppenga, 2009). Lethargy, wing droop, leg paresis, changes in phonation, head tilt, ataxia, blindness, circling, head tremors, and seizures are notable signs related to the nervous system dysfunction. GI signs include regurgitation and decreased motility of the upper GI tract (esophagus, proventriculus, and ventriculus) resulting in impaction and greenish diarrhea that stains feathers around the vent (Dumonceaux and Harrison, 1994). Anemia as a result of increased erythrocyte fragility and delayed maturation is observed in affected birds. Whole blood lead concentration does not always correlate with the severity of clinical signs.

Emaciation and hydropericardium were the major gross lesions in experimentally dosed bald eagles (*H. leucocephalus*) (Pattee *et al.*, 1981). Pale musculature or viscera consistent with anemia, renal or visceral gout, air sacculitis, muscle and fat atrophy, and splenomegaly have been reported in various avian species (Locke and Thomas, 1996). Bile stasis leading to an engorged gallbladder, dark-green viscous bile, bile-stained gastric and intestinal mucosa, and a greenish appearance to the liver have been reported in raptors (Locke and Thomas, 1996).

Measurement of whole blood lead concentration in live birds or in the liver and kidney of dead birds is employed for diagnosis and is widely available. A whole blood lead concentration of 0.20 ppm (20 µg/dL) or greater is considered to be consistent with lead exposure and/or intoxication. Because lead associates with red blood cells, serum and plasma are not appropriate for testing. Generally, 4 ppm wet weight or greater lead concentration in the avian liver and kidney is likely to be significant (Puschner and Poppenga, 2009). Radiographic identification of metallic objects in the GI tract is helpful, but their absence does not preclude lead exposure because the source may not be radio-dense (e.g., contaminated soil). Other biomarkers of lead exposure, such as aminolevulinic dehydratase activity, blood zinc protoporphyrin concentration, and free erythrocyte protoporphyrin concentration, are used but not widely available.

Multiple decontamination approaches have been utilized, including saline lavage, administration of size-appropriate pieces of grit to promote excretion, use of laxatives such as mineral oil or psyllium, and use of cathartics such as sodium sulfate. Endoscopy can be used to remove lead particles entrapped in proventricular or ventricular folds (Samour and Naldo, 2005). Various chelation therapies are employed for the treatment of lead intoxication. Several chelators can effectively bind lead, including CaNa<sub>2</sub>EDTA, succimer, D-penicillamine, and British anti-lewisite (BAL). Succimer and CaNa<sub>2</sub>EDTA are currently the chelators of choice, although no veterinary-approved forms are available. Symptomatic and supportive care including seizure control, fluid and electrolyte therapy for birds experiencing diarrhea, and vitamin supplementation may further aid in patient stabilization and therapy.

## Zinc

Galvanized cage wires and accessories, pennies minted after 1982, and contaminated soil, water, and feed are some of the documented sources of zinc associated with avian toxicosis (Reece *et al.*, 1986; Howard, 1992; Lloyd, 1992; Romagnano *et al.*, 1995; Puschner and Poppenga, 2009). In immature chickens and turkeys, dietary concentrations between 800 and 4000 ppm (mg/kg) of zinc oxide, sulfate, or carbonate have been correlated with reduced growth and muscular dystrophy (Sell *et al.*, 1994).

Zinc is absorbed in the proventriculus and small intestine. The exact uptake mechanism is unknown; however, it is a carrier-mediated step of facilitated diffusion and is not energy dependent (Menard and Cousins, 1983). Intestinal zinc absorption is affected by dietary concentrations and form of zinc, the presence of other minerals in the intestinal lumen, the availability of zinc

chelating agents in the diet, and the synthesis of zinc carrier molecule(s) in the mucosal cells of small intestine (Song, 1987). Post-absorption, zinc is distributed in the pancreas, liver, kidneys, bones, muscles, brain, retinas, intestinal mucosa, and skin, where it binds to metallothionein, especially in the pancreas, liver, kidneys, intestinal mucosa, and brain. Turnover and secretion of zinc by the pancreas, bile, and gastroduodenal secretions leads to its excretion in feces.

Specific mechanisms underlying the toxic effects of zinc are not established; however, the direct and indirect toxic effects of zinc are observed on the GI tract, liver, kidneys, pancreas, red blood cells, and brain. The consequences of high intracellular zinc concentrations lead to increased cellular reactive oxygen species production, reduced cellular ATP concentrations, and loss of mitochondrial membrane potential (Dineley *et al.*, 2003).

Zinc intoxication has been reported in several avian species. In experimentally inoculated cockatiels, 32 mg/week of pure zinc powder or galvanized coating removed from welded wire mesh was observed to cause severe illness and death within 2 weeks. In addition, a dose of 2 mg/week for 6 weeks induced chronic signs marked by dullness, weight loss, and intermittent excretion of greenish droppings (Howard, 1992).

In acute cases, neurologic signs, corrosive lesions in the GI tract leading to associated disturbances, and injury to the liver, kidneys, and pancreas may be observed. In chronic cases, erythrocyte abnormalities and anemia may be commonly observed, and these have been thought to be a result of oxidative damage to the erythrocyte hemoglobin and cell membrane proteins (Luttgen *et al.*, 1990). Clinically affected birds may exhibit signs of lethargy, anorexia, polyuria, polydipsia, hematuria, hematochezia, regurgitation, pallor, dark or bright green diarrhea, foul-smelling feces, paresis, seizures, and sudden death (Howard, 1992; Lloyd, 1992; Romagnano *et al.*, 1995; Christopher *et al.*, 2004). Various degrees of dehydration and caudal staining of the vent with fecal/urate containing material as a result of diarrhea have been noted in some cases. Neurological signs such as intermittent head bobbing and ataxia may occasionally be observed as well. In ducks with severe zinc-induced hemolysis, a functional iron deficiency may impair the effectiveness of the erythropoietic response and contribute to death (Christopher *et al.*, 2004).

Common findings on gross examination of birds that have died from zinc toxicosis include greenish, mucoid feces in the ileum, colon, or cloaca and muscle wasting, especially of the pectoral muscles. Occasionally, the liver or kidneys are slightly enlarged. No other consistent lesions are usually noted on gross examination. Microscopically, pancreas may reveal severe apoptosis, characterized by cell remnants appearing as acidophilic bodies containing pyknotic nuclei accompanied by a

loss of normal acinar architecture, necrotizing pancreatitis, the presence of hyaline bodies and other electron-dense debris, cellular atrophy and necrosis of individual acinar cells, and interstitial fibrosis (Wight and Dewar, 1986; Zdziarski *et al.*, 1994). Hemosiderosis in the liver, accompanied by hepatic biliary retention and multifocal, necrotizing hepatitis, may be observed (Howard, 1992; Puschner and Poppenga, 2009). Lesions in the kidneys include varying degrees of acute tubular necrosis, occasionally with secondary renal or visceral gout, and moderate interstitial nephritis in addition to nephrosis. GI lesions include intestinal hemorrhage, hemorrhagic enteritis, hemorrhagic ventriculitis, and ventricular koilin degeneration (Puschner *et al.*, 1999).

Diagnosis of zinc intoxication can be challenging. The patient history may hold clues to exposure (e.g., exposure to a new cage, feeding and/or watering utensils, or metallic toys). Clinical signs may not always be specific; therefore, a thorough clinical examination, construction of a relevant list of differentials, radiography, diagnostic sampling of the serum/plasma in live birds, blood smear evaluation, and collection of the liver and kidney in the case of dead birds are vital. The absence of radiographically evident metal densities in the GI tract does not rule out zinc toxicosis in the differential diagnosis because some particles might not be dense enough to appear. A necropsy with complete histologic evaluation should be performed on all birds that have died of a potential metal toxicosis. In live birds showing clinical signs suggestive of zinc poisoning, serum and plasma samples are considered suitable for zinc determination (Kosman and Henkin, 1979). For most laboratories, sample volumes of 50–100  $\mu$ L are sufficient for analysis. Special care must be taken to avoid contact with rubber products that can be a source of zinc (e.g., rubber-topped tubes) and hemolysis, which may also increase zinc concentrations. In addition, zinc concentrations in plasma collected from psittacines show significant diurnal variation, with the highest concentrations detected in morning samples (Rosenthal *et al.*, 2005). For most psittacines, a physiologic, nontoxic zinc concentration in serum or plasma is 2 ppm (0.2 mg/dL) or less. Cockatoos and eclectus parrots tend to have higher physiologic concentrations of zinc in serum and plasma, with acceptable nontoxic concentrations of up to 3.5 ppm (0.35 mg/dL) for cockatoos and up to 2.5 ppm (0.25 mg/dL) for eclectus parrots (Puschner *et al.*, 1999). An assessment of erythrocyte morphology can aid in the diagnosis of zinc poisoning in birds. Observed abnormalities include a greater number of immature red blood cells; hypochromasia; poikilocytosis; and nuclear abnormalities such as fusiform, elongated, and irregular nuclei (Christopher *et al.*, 2004). Postmortem determination of liver or kidney zinc concentrations is useful. Most companion birds have acceptable liver zinc concentrations of 30–70 ppm (mg/kg) wet weight (Puschner *et al.*,

1999), and liver zinc concentrations of up to 100 ppm (mg/kg) expressed as wet weight are considered non-toxic. Toxic concentrations of zinc in liver tissue typically exceed 100 ppm (Smith, 1995).

If a radio-dense zinc object is identified by radiography, adequate treatment options such as lavage, use of emollient laxatives or cathartics, endoscopy, or surgery can be utilized. Removal of the zinc source results in the rapid decline of body concentrations. If immediate removal is not possible or delays are expected, reducing the further absorption of zinc in the GI tract may be achieved by administering antacids such as calcium carbonate (Van der Merwe and Tawde, 2009). Complete removal of the source of zinc in a timely manner may not always be possible. Financial considerations, very small particles not amenable to endoscopic removal, and uncooperative patients with weak physiological status hindering anesthesia may all potentially decrease treatment options. Chelation of zinc is possible with  $\text{CaNa}_2\text{EDTA}$ , although its efficacy and safety in the treatment of zinc intoxication in birds have not been evaluated.

## Mercury

Natural (e.g., volcanoes or weathering rocks) and anthropogenic sources such as coal combustion or industrial activities contribute to mercury accumulation in the environment. Methylmercury (MeHg) presents the most significant risk for intoxication in birds, particularly wild piscivorous species. Methylation of inorganic mercury by aquatic anaerobic organisms leads to biomagnification by microbes and plankton and subsequent accumulation in fish, which in turn can then be a significant source of exposure for fish-eating birds such as loons, mergansers, and bald eagles. Consumption of MeHg-containing diets at environmentally realistic concentrations has been demonstrated to cause behavioral, neurological, hormonal, and reproductive changes in birds, fish, and mammals (Scheuhammer *et al.*, 2007). Interestingly, subtle vocalization changes in selected mercury-exposed avian species have been noted by the analysis of spectrographic bird-songs, including a lower diversity of note types and lower tonal frequencies than in songs of birds at control sites (Hallinger *et al.*, 2010).

MeHg is metabolized and excreted more slowly than other organomercurials (Stickel *et al.*, 1977). The lipophilic nature of MeHg allows it to penetrate the blood-brain barrier, where the majority of toxic effects are expressed. Clinical signs due to mercury intoxication are similar in multiple species of birds and are characterized by reduced food intake, weakness in wings and legs, with difficulty flying, walking, and standing (Borg *et al.*, 1970; Fimreite and Karstad, 1971). Affected birds are unable to coordinate muscle movements (Tejning,

1967). Appearance of these symptoms typically leads to death, even if the source of exposure is removed. Feeding MeHg-contaminated seed at 2 or 3 ppm for 12 weeks to adult breeding pheasants (*Phasianus colchicus*) produced a significant increase in the laying of shell-less eggs and a decrease in mean egg weight. Decreased hatchability due to early embryonic mortality and an increased number of unfertilized eggs were observed compared to controls. Mating behavior, egg production, and mortality were not affected, nor was any effect on food consumption or the nervous system observed. Unhatched eggs contained 0.5–1.5 ppm mercury, and the livers of experimental birds contained approximately 2 ppm mercury (Fimreite, 1971). Spinal cord degeneration due to MeHg toxicity has been noted in birds (Scheuhammer, 1987); however, there are considerable differences in sensitivity to toxic effects of MeHg. Concurrent administration/exposure to selenium may act to form stable, nontoxic mercury-selenium complexes, preventing the attachment of mercury with sulfhydryl groups of enzymes and other bioligands (Sugiura *et al.*, 1976), thereby acting as a modifying factor in the toxicity of MeHg. Mercury exposures that might otherwise produce toxic effects are therefore counteracted by selenium, particularly when Se:Hg molar ratios approach or exceed 1 in marine or freshwater fish (Ganter *et al.*, 1972; Luten *et al.*, 1980).

Kidney MeHg concentrations of 40 ppm and higher have been associated with mortality in a number of bird species (Finley *et al.*, 1979); however, elevated tissue concentrations without overt signs of toxicity have been observed in experimental birds. The diagnosis of mercury intoxication therefore should not be based on tissue concentrations of mercury alone and should necessarily address corresponding selenium concentrations, the presence or absence of compatible clinical signs and necropsy findings, and the absence of other infectious or noninfectious causes. Selected chelators such as dimercaprol (BAL) and D-penicillamine (Dumonceaux and Harrison, 1994) can be used in birds to chelate Hg. Selenium and vitamin E supplementation can additionally be utilized to reduce the toxic potential of mercury before the appearance of expected signs.

## Iron

An increasing incidence of hepatic iron storage disease has been recorded in captive birds during approximately the past four decades, particularly associated with frugivorous species of the families Paradisaeidae (birds-of-paradise), Ramphastidae (toucans), and Sturnidae (starlings) (Sheppard and Dierenfeld, 2002). Often described as hemosiderosis due to the deposition of a ferritin-containing iron complex (hemosiderin), these elevated iron stores in



the liver and other organs may sometimes lead to icterus, ascites, and accompanying liver pathology. However, non-symptomatic hemosiderosis has been reported as well (Taylor, 1984; Ward *et al.*, 1988; Borch-Johnsen *et al.*, 1991). Dietary factors affecting iron metabolism and/or genetically regulated increased iron uptake in sensitive species may lead to drastically increased iron concentrations in the liver, sometimes as high as 5517 mg/kg mean wet weight ( $N=22$ ,  $SD=3797$ ) in captive Paradisidae (Dierenfeld *et al.*, 1994). In captive birds, evolutionary physiological mechanisms compensating for low bioavailability of iron and the supplementation of iron in formulated diets may contribute to excessive storage (Sheppard and Dierenfeld, 2002). In addition, nutritional interactions in mixed diets and greater bioavailability of dietary iron further complicate the issue. Dietary iron requirements of many sensitive avian species are not known, which makes the formulation of a low-risk diet especially challenging. A study found a significant correlation between hepatic hemosiderosis with age and time in captivity, suggesting a careful revision of commercial diets for sensitive and endangered species in captivity (Pereira *et al.*, 2010).

Although hemosiderosis has been reported in Galliformes, iron storage disease has not been reported. Many commercial diets intended for captive birds have been formulated using the nutritional requirements of poultry. These do not take into account the primarily fruit- and insect-based low-iron-containing diet of other sensitive avian species. In addition, the mineral, carbohydrate, and sugar profiles, vitamin concentrations, polyphenolic contents, and the use of animal byproducts significantly affect the nutritional interactions and bioavailability of iron. Absorption of iron from animal byproducts (heme-based iron sources) may be three times higher than that from non-heme products containing equivalent iron concentrations. Thus, higher dietary iron concentrations, increased bioavailability, and absorption in sensitive species form the major etiological factors of iron storage disease. A diet containing 50–100 mg/kg iron on a dry matter basis has been recommended for sensitive avian species (Sheppard and Dierenfeld, 2002).

Increasing cellular iron concentrations leads to lysosomal injury and release of ionic iron, causing oxidative damage to membranes and proteins. Liver, heart, and spleen are most commonly affected (Randell *et al.*, 1981; Gosselin and Kramer, 1983; Kincaid and Stoskopf, 1987). Fibrotic changes in the liver affect its function, commonly leading to ascites, hypoalbuminemia, and icteric symptoms. Clinically, dyspnea, abdominal distension, weight loss, and depression are major signs. Radiographically, the enlargement of liver, heart, and spleen may often be observed. Hematology and serum biochemistry are reportedly of less value, although elevations in liver enzymes may be noted. Serum ferritin reference ranges are not available, although transferrin

concentrations have been used. Various inflammatory, infectious, hemolytic, and hepatic diseases may elevate ferritin concentrations. Serum iron and total iron binding capacity are poor indicators of body or liver iron stores. A liver biopsy is often needed to confirm iron storage disease antemortem.

Treatment for iron storage disease relies on multiple approaches. In valuable birds, chelation therapy using deferoxamine (100 mg/kg, q24h, subcutaneously) (Cornelissen *et al.*, 1995) can be utilized, along with low-iron-containing diets. CaEDTA and BAL will chelate iron as well. Although stressful, phlebotomy of affected birds will force the body to use stored iron. If sufficient damage has occurred to internal organs, these interventions may be of little therapeutic value. Prophylactically, intermittent feeding of very low iron-containing diet may be useful; however, caution has to be exercised not to induce iron-deficiency anemia in birds. The addition of natural chelators such as phytates, fiber, and tannins may be of value, and the addition of tea leaves has been experimentally found to be useful (Seibels *et al.*, 2003). However, questionable consistency of chelation and the potential of chelating multiple minerals in the diet make this approach somewhat risky, and it needs further investigation and refinement.

## Selenium

Although rare, avian selenium toxicosis typically manifests as decreased egg production and hatchability, reduced weight gain and feed efficiency, embryonic deformities, and death. Consumption of water from irrigation drains containing excess selenium by aquatic birds and accidental inclusion of excess selenium in feed have been reported to cause selenium intoxication in avian populations (Ohlendorf *et al.*, 1986).

The bioavailability and relative toxicity of selenium depend on its chemical form and solubility. Elemental selenium is far less soluble and toxic than the selenite and selenate forms. Organic selenium forms (e.g., selenomethionine) are more effective in raising tissue selenium concentrations than are the inorganic selenite or selenate salts; although selenite was found to depress growth performance in chicks more than selenomethionine fed at equal concentrations of 15 mg/kg BW (Lowry and Baker, 1989). In mallard ducklings, both D and L forms of purified selenomethionine were more toxic than selenized yeast (containing approximately 65% L-selenomethionine) (Heinz *et al.*, 1996). Many trace elements, including mercury, arsenic, antimony, bismuth, cadmium, silver, and tungsten, can affect selenium metabolism and toxicity (Rahim *et al.*, 1986; Sell *et al.*, 1994).

A detailed case report of selenium intoxication in breeding ring-necked pheasants describes 12% mortality in the

first week of feeding selenium-containing feed (9.3 mg/kg feed). Necropsy of dead birds revealed colorless fluid around the heart and friable livers. Histologically, degenerative cardiomyopathy, vacuolar degeneration of hepatocytes, and centrilobular hepatic necrosis were noted. Feed replacement returned egg production to normal in 10 days. Post-exposure, 10% of the chicks that hatched had deformed beaks and abnormal eyes. Fifty percent of all embryos that developed in eggs had deformities and died within shells. Another case report involving consumption of irrigation drain water by aquatic birds described heart defects, hydrocephaly, abnormal legs and wings, and size variations in the liver (Ohlendorf *et al.*, 1986). In controlled studies, mallards (*Anas platyrhynchos*) fed selenomethionine and increasing concentrations of sodium selenite developed comparable lesions as described previously. In addition, selenomethionine was concluded to be considerably more teratogenic and embryotoxic than sodium selenite, possibly due to higher uptake (Hoffman and Heinz, 1988).

## ENVIRONMENTAL TOXICANTS

### Pentachlorophenol

Pentachlorophenol (PCP) is used primarily as a wood preservative. Poultry have been exposed to sawdust and shavings from PCP-treated wood (Brown *et al.*, 1997). Adverse effects associated with PCP include reductions in growth rates, kidney hypertrophy, and decreased humoral immune response (Stedman *et al.*, 1980; Prescott *et al.*, 1982). PCP exposure can also result in an off-taste to eggs and meat as a result of degradation of chlorophenols to chloroanisols (Frank *et al.*, 1983).

### Petroleum

Exposure of avian wildlife to crude oil is a significant cause of morbidity and mortality. Although oil spills involving large tanker accidents such as the grounding of the *Exxon Valdez* in Alaska receive most of the public's attention, other scenarios related to oil drilling and production, off-loading of oil from tankers, production, processing, and refining of oil, road transport, and improper disposal of waste oil and petroleum products result in considerable wildlife exposure (Jessup and Leighton, 1996). Because oil floats on the surface of water, birds that live on water or dive through water for food are more likely to be exposed in water spills. Marine birds and bird species that utilize environments near to shore are also likely to be exposed to oil if spills reach shorelines. Raptors such as peregrine falcons that feed on

other birds can be exposed to oil from contaminated prey (Zuberogoitia *et al.*, 2006).

Crude oil and petroleum products are complex mixtures of chemicals, including a variety of aromatic and aliphatic compounds. The toxicity of crude oils and petroleum products varies depending on their chemical compositions. Another complicating factor in assessing the toxicity of crude oil is the fact that soon after environmental release, the process of "weathering" occurs, which changes its chemical and physical properties.

Crude oil and other petroleum products affect birds in several ways. First, loss of insulating properties of the feathers results in rapid hypothermia. Oiled birds lose their ability to fly, and they frequently die from starvation, exhaustion, or drowning (Friend and Franson, 1999). Second, crude oil and other petroleum products are irritating to skin, mucous membranes, and the respiratory tract. Aspiration of oil into the respiratory tract and lungs can cause aspiration pneumonia. Third, systemic absorption of chemicals found in crude oil or petroleum products causes adverse effects on several organ systems, including reproductive, hematopoietic, nervous, immune, and hepatobiliary systems. Lastly, petroleum is extremely toxic to bird embryos.

Diagnosing petroleum intoxication as a cause of death can be challenging. There are no characteristic gross or histopathologic lesions specific to petroleum intoxication. A diagnosis is based on a history or evidence of exposure and compatible clinical and postmortem findings. Exposed birds, especially those with white or light-colored feathers, are easily identified because of the persistence of oil on feathers and skin. Birds are often emaciated, oil may be present in the respiratory or GI tracts, there may be congestion along the intestines, and the salt glands may be swollen (Friend and Franson, 1999). Feathers, skin, or organs of birds that do not have external evidence of exposure can be analyzed for the presence of chemicals typically found in crude oil or petroleum products (Jessup and Leighton, 1996).

### Cyanide

Cyanide (hydrocyanic acid) is a rapidly acting cellular poison. Poisoning most often occurs following ingestion of a cyanide salt or inhalation of hydrogen cyanide gas. Avian wildlife intoxications have occurred following exposure to cyanide used in heap leach and carbon-in-pulp mill gold or silver mining processes (Friend and Franson, 1999). Birds are most often intoxicated by ingesting cyanide-contaminated water. Between 1986 and 1995, 3000 cyanide-related mortalities involving 75 species of birds were reported to the National Wildlife Health Center (Friend and Franson, 1999). Most mortality events occur in the spring and fall as migratory birds

pass through mining areas. Large-scale cyanide spills have caused widespread contamination of rivers in Europe and South America (Koenig, 2000).

Cyanide binds to ferric ( $\text{Fe}^{+3}$ ) iron of mitochondrial cytochrome oxidase. This enzyme mediates the transfer of electrons to molecular oxygen, which is the last step in oxidative phosphorylation (Delaney, 2001). Cells are unable to use oxygen, resulting in tissue anoxia, increased anaerobic metabolism, and rapid development of lactic acidosis.

The acute oral toxicity of sodium cyanide has been studied in several bird species.  $\text{LD}_{50}$ s ranged from 4.0 to 21 mg/kg BW (Wiemeyer *et al.*, 1986).

Cyanide is a rapidly acting toxicant; most intoxicated birds are found dead. Grossly, dead animals often have bright red blood and multiorgan congestion. A diagnosis is based on the history of exposure and measurement of cyanide in blood, heart, liver, or brain. Samples should be stored and shipped frozen to avoid the loss of cyanide before analysis. Denying birds access to cyanide-contaminated water is the best preventive approach.

## TOXIC GASES

### Ammonia

High ambient air concentrations of ammonia (50–75 ppm) reduce feed consumption, growth rate, and egg production in poultry (Deaton *et al.*, 1986). Ammonia readily dissolves in water to form ammonium hydroxide, which is an alkaline irritant. Thus, contact with the moist mucous membranes of the eyes and respiratory tract results in corneal and epithelial cell damage. Toxic air concentrations cause keratoconjunctivitis, corneal ulceration, blindness, photophobia, tracheitis, tachypnea, and dyspnea. Prolonged exposure can result in increased mucous secretion and hyperplasia of the bronchiolar and alveolar epithelium. Ambient ammonia air concentrations should be greater than 25 ppm for poultry (Brown and Julian, 2003).

### Carbon monoxide

CO is an odorless, colorless, lighter-than-air gas that can reach toxic concentrations as a result of incomplete combustion of hydrocarbon-based fuels. Toxic concentrations of CO most often result from use of defective gas catalytic or open-flame brooders and furnaces or internal combustion engines in poorly or unventilated spaces. CO acts by competing with oxygen for a number of proteins, including hemoglobin. The affinity of hemoglobin for CO is approximately 250 times greater than that for oxygen.

Formation of carboxyhemoglobin reduces the ability of red blood cells to carry oxygen, and the oxygen dissociation curve is shifted to the left, resulting in tissue hypoxia. Affected birds exhibit drowsiness, labored breathing, weakness, and ataxia, with seizures occurring before death (Brown and Julian, 2003). Birds may be found dead. Carboxyhemoglobin causes the blood to have a bright red appearance. Carboxyhemoglobin concentrations of 20% are associated with some motor impairment; death occurs when blood carboxyhemoglobin concentrations approach 60–70% (Osweiler *et al.*, 1985). Ambient CO concentrations of 600 ppm for 30 min cause clinical signs in chickens; concentrations of 2000 ppm or greater are lethal within 1.5–2 h (Brown and Julian, 2003). Sublethal exposure to CO in poultry causes stunting.

Diagnosis of CO intoxication is based on a history compatible with CO production, compatible clinical signs, and measurement of ambient air CO and blood carboxyhemoglobin concentrations. Treatment is generally limited to provision of fresh air or oxygen; pet birds can be placed in oxygen cages.

Other gases, such as hydrogen sulfide and carbon dioxide, generally do not present significant intoxication risks for birds. Interestingly, chickens are less sensitive to hydrogen sulfide than are humans or dogs (Brown *et al.*, 1997).

### Polytetrafluoroethylene: Teflon

Polytetrafluoroethylene (PTFE) is a synthetic polymer that is widely used as a nonstick surface in cookware. It is also used in self-cleaning ovens and as a coating on heat lamp bulbs. Heated to high temperatures ( $\geq 280^\circ\text{C}$ ), PTFE releases toxic pyrolysis products that can cause rapid death of birds. There are no reported toxicities associated with the proper use of coated cookware because temperatures do not reach levels at which pyrolysis occurs. However, cookware without contents can reach critical temperatures within minutes.

Although all birds are likely to be susceptible to intoxication, most spontaneous cases involve pet birds such as cockatiels, parrots, finches, and budgerigars (Blandford *et al.*, 1975; Wells, 1983; Stoltz *et al.*, 1992). There is one case report in which broiler chicks were intoxicated from heat lamp bulbs coated with PTFE (Boucher *et al.*, 2000) and another suspected intoxication of wild birds associated with industrial activity (Pennycott and Middleton, 1997). Quail have been exposed experimentally (Griffith *et al.*, 1973).

PTFE pyrolysis products contain particulates of respirable size ( $<1\ \mu\text{m}$  diameter) that can penetrate to the alveoli (Wells *et al.*, 1982). The particulates are themselves toxic, but they also serve as a vehicle for other potentially damaging chemicals, including hydrogen fluoride,

carbonyl fluoride, and perfluoroisobutylene (Wells and Slocombe, 1982). The fumes are acidic and cause direct damage to cell membranes of the lungs. However, it appears that the particles are necessary for intoxication to occur because the gas phase of PTFE fumes alone does not cause damage (Seidel *et al.*, 1991). Lung damage is believed to be due to direct irritation and oxidative damage (Wells *et al.*, 1982; Johnston *et al.*, 1996).

Precise toxicity information is not available. Exposure of budgerigars to PTFE pyrolysis products for 9 min or longer resulted in severe clinical signs, death, and significant pulmonary lesions in 31 of 32 birds (Wells *et al.*, 1982). Although there is little comparative toxicity information among avian species, budgerigars were more sensitive than quail in one experimental study (Griffith *et al.*, 1973).

After exposure, the onset of clinical signs is rapid. Eyelid blinking is an early sign and may be related to a direct irritant effect on the conjunctiva and cornea (Wells *et al.*, 1982). Eyelid movements could also be secondary to hypoxia-induced somnolence. Tachypnea, dyspnea, anxiety, cage wire biting, incoordination, and inability to stand may be due to hypoxia secondary to lung damage. In many cases, the bird may be found dead with no other clinical signs noted.

Grossly, the lungs are severely congested and hemorrhagic (Wells and Slocombe, 1982). Histologically, there is extensive, severe, necrotizing, and hemorrhagic pneumonitis. Amorphous, elongate particles may also be noted (Wells and Slocombe, 1982).

A diagnosis is generally based on a history of exposure and characteristic postmortem lesions. Currently, there is no analytical test available to confirm exposure to the pyrolysis products. The rapidity of onset of severe signs and subsequent death most often precludes treatment. Awareness of the hazard and avoiding housing birds near PTFE coatings is the best prevention.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Given the number and diversity of avian species and their environments and the diversity of chemicals to which they are exposed, assessing the impact of toxicants on individuals and populations is a daunting task. Toxicants can have direct toxic effects or, more subtly, indirect effects on the availability of important prey items. In addition, birds are commonly exposed to toxicants that accumulate in their prey (e.g., mercury, lead, and anticoagulant rodenticides). Although the diagnosis of acute intoxication is frequently straightforward, the challenge in avian toxicology is twofold. First, it is

sometimes difficult to determine the sublethal impact of more chronic exposure to toxicants on bird health. For example, does exposure to methylmercury predispose susceptible birds to sublethal effects on reproduction or altered fledgling success? Second, although the impact of toxicant exposure on individuals might be easily determined, it is more difficult to assess whether individual effects translate into adverse effects on populations. There is no doubt that many raptor species are exposed to anticoagulant rodenticides. However, the population impacts of such exposures are largely unknown. Better defining the effects of chronic, low-level toxicant exposures and toxicant effects on the long-term stability of avian populations are future challenges.

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# Aquatic toxicology

Robert W. Coppock and P.N. Nation

## INTRODUCTION

Aquaculture is the production of aquatic animals for human food. In aquaculture, aquatic animals are grown immersed in the aquatic environment. The water may be a sheltered oceanic bay containing pens of aquatic organisms, or the aquatic organisms may be raised in inland ponds or in a tank system. In colder climates, a recirculation tank system in a heated building may be used. The more contained the aquatic rearing system, the more diligent must be monitoring and control of the artificial ecosystem.

Intoxication of fish can be acute, subacute, or chronic. Chemical causes of disease in aquatic organisms are generally linked to ambient water and food because these are the primary pathways of toxic substances to animals in aquaculture systems. Chemical-linked food safety issues can occur if chemical contamination of edible aquatic organisms occurs. The toxicity of a specific substance can vary between fish species and can change with water temperature, water pH, and ion composition (Wlasow *et al.*, 2010). In recirculation systems, waste materials and microbial degradation products can reach toxic levels. The interactions between infectious diseases and chemically induced disease are complex (Morley, 2010). Chemical intoxication and other environmental stressors can increase the susceptibility of aquatic organisms to infectious diseases. The predisposing causes of infectious disease can be overlooked because they may be subtle or unrecognized. The toxicology of the water column can be different from that of the sediment, and these variations in the aquatic environment present different issues to various segments of the aquaculture industry (Rudolph *et al.*, 2009).

This chapter focuses on aquatic toxicology as it relates to the culture of fish. However, the principles and many of the specifics that are discussed apply to most aquatic organisms, cultured or not.

## BACKGROUND

Aquaculture is an expanding and continually developing industry. Fish are cultured in some form of confinement such as a pen, pond, or tank. Cultured fish are usually dependent on humans for part or all of their feed and can be entirely dependent on humans for maintenance of their ecosystem. Commercial feed is generally presented in a granular or pelleted form and contains a binding agent. Inland aquaculture often requires at least some mechanical management of the aquatic environment. Large capital investment and high operational costs are incentives to maximize stocking densities in aquaculture systems. Increased stocking densities increase the degree of sophistication required for water management. Upstream users can contaminate the source of water for aquaculture, as can floods and chemical spills that find their way into water bodies.

## WATER SAFETY

Water safety is a particular concern with large commercial rearing operations. Unsafe water kills more fish in

tanks and ponds than all other causes of disease. In the wild, fish generally seek water that meets their needs for pH, dissolved oxygen, and other necessities. Fish in confinement are unable to do this. Fish can also directly affect safety of the water in which they live, and their metabolic activity can move water parameters from safe to unsafe. Sensitivity to parameters affecting water safety in aquaculture varies with age of the fish and stage of development (Finn, 2007). The factors in water safety for both wild and captive-reared fish are interactive, and changing one parameter always affects other factors. The pH of water is important in affecting the solubility of metals (Finn, 2007). Lowering the pH generally decreases the bioavailability of metals by increasing complexes with organic matter in the water. Temperature of the water is important, and the ideal temperature can vary with life stage and genera.

## Nitrogen cycle

The nitrogen cycle in aquatic systems is important (Boyd and Tucker, 1998). An excellent review on the geobiochemistry of the nitrogen cycle has been written (Hargreaves, 1998). The nitrogen cycle transforms organic nitrogen, ammonia, and other forms of nitrogen to nitrogen gas. The most common ionic forms of nitrogen are ammonium ( $\text{NH}_4^+$ ), nitrite ( $\text{NO}_2^-$ ), and nitrate ( $\text{NO}_3^-$ ) (Camargo *et al.*, 2005). Sources of these ions are atmospheric deposition, presence in surface and groundwater, nitrogen fixation, and degradation of organic matter. Important sources of nitrogen in the aquatic system are feedstuff residues, feces, and ammonia excreted from aquatic animals. The nitrogen cycle in an aquatic system is primarily regulated by biota. The organisms regulating the different steps in the aquatic nitrogen cycle are remarkably independent, and the products from the different steps can accumulate in the system. Species of *Nitrosomonas* and *Nitrobacter* oxidize ammonia to nitrite. Following this, oxidation of  $\text{NO}_2^-$  to  $\text{NO}_3^-$  occurs, and species of *Nitrobacter* are the primary organisms that accomplish this. Species of *Nitrosomonas* and *Nitrobacter* are dissimilarly affected by oxygen tension, pH, temperature, etc. Species of *Nitrobacter* are the most sensitive, and when they are disadvantaged, the level of  $\text{NO}_2^-$  increases. The end step in the nitrogen cycle is reduction, with production of nitrogen gas ( $\text{N}_2$ ) released into the atmosphere. The most toxic compounds in the aquatic nitrogen cycle are ammonia and  $\text{NO}_2^-$ . Changes in the nitrogen cycle can also be seasonal and/or related to weather conditions. For example, nitrate poisoning (brown blood disease) in the southern United States is more common in the fall and spring (Durborow *et al.*, 1997). When the concentration of nitrite is less than  $1\text{ }\mu\text{mol}$ , the water is generally considered unpolluted (Jensen, 2003).

## Ammonia

The economics of tank-rearing of fish requires high stocking densities. Recirculation systems have densities as high as 0.3 kg of fish/kg of water. Estimated ammonia production by fish is 0.02 kg of ammonia nitrogen produced for each kilogram of feed fed (Masser *et al.*, 1999). Total ammonia nitrogen should be monitored in the aquatic system because ammonia is the primary nitrogen waste excreted by freshwater fish and is the most toxic form. At a given pH of the water, ammonia ( $\text{NH}_3$ ) is in equilibrium with  $\text{NH}_4^+$ . The unionized ammonia can radially diffuse across cell membranes. The toxicity of ammonia is dependent on the pH of the water, water temperature, and the species of aquatic animal. The best measurement to predict water safety is total ammonia nitrogen (TA-N), which is the sum of  $\text{NH}_3$  +  $\text{NH}_4^+$  forms. The pK of ammonia is approximately 9.2. At a pH of approximately 9.2,  $\text{NH}_3$  is in equilibrium with  $\text{NH}_4^+$  +  $\text{OH}^-$ . Lowering pH below the pK increases the concentration of ionized  $\text{NH}_4^+$ .

Fish excrete 40–60% of ingested nitrogen within 24 h (Ip and Chew, 2010). The liver is responsible for the majority of ammonia production. Most of the ammonia comes from catabolism of  $\alpha$ -amino acids that are channeled into gluconeogenesis and the citric acid cycle. Ammonia can be converted to less toxic forms, such as urea and uric acid, but requires energy. Most fish species excrete excess nitrogen from deamination of amino acids as ammonia (Wilkie, 2002). Magadi tilapia (*Tilapia grahami*) excrete excess nitrogen as urea by using the ornithine urea cycle. Fish metabolize excess purines through uricolysis and excrete the nitrogen as urea. The gills excrete ammonia down a concentration gradient. The excretion of ammonia varies between freshwater and saltwater fish, and the mechanisms are not well established. Saltwater fish are more sensitive to ammonia toxicity than are freshwater fish (Randall and Tsui, 2002). There is evidence that minimal energy is used in the ammonia secretion process and transporter proteins facilitate the diffusion process. Increasing pH of the ambient water decreases excretion of ammonia. Most of the ammonia in fish blood exists as ammonium ion because the pH of blood is approximately 2 pH units below the pK of ammonia (~9.5). Increased ammonium in fish increases glycolysis in cytosol and decreases the TCA cycle in the mitochondria. The ammonium ( $\text{NH}_4^+$ ) ion at high concentrations can substitute for  $\text{K}^+$  in ion transporters and disrupt electrochemical gradients. Physical activity, feeding, and stress due to increased cortisol levels increase blood ammonia (Randall and Tsui, 2002). Increased blood ammonia reduces physical activity. The mechanisms of ammonia toxicity in fish have not been elucidated. Amino acid metabolism is disrupted (Ariello *et al.*, 1984; Smutna *et al.*, 2002). There



appear to be metabolic dysfunctions in the liver and links to disruption of neurotransmitters and electrochemistry in skeletal muscle (Randall and Tsui, 2002). Increasing water ammonia decreases swimming ability (Wicks *et al.*, 2002). The decrease in swimming ability was linked to depolarization of white muscles, and increasing water calcium ions ameliorated ammonia toxicity. Feeding fish appears to provide some protection from ammonia toxicity (Wicks and Randall, 2002). Increasing ammonia in ambient water increases oxidative stress in the gills and disrupts ion transport (Ip and Chew, 2010). The chronic toxicity of ammonia at a pH of approximately 7.93 and  $\text{NH}_3$  concentrations of 0.0,  $0.215 \pm 0.03$ , and  $0.423 \pm 0.163$  ppm, respectively, was studied in rainbow trout (*Oncorhynchus mykiss*) fingerlings (Daoust and Ferguson, 1984). Increased blood ammonia can interfere with neurotransmitters. Clinical signs of intoxication were not observed in fish exposed to 0.0 and 0.215 ppm  $\text{NH}_3$ . The water was maintained at optimal oxygen level. In fish exposed to approximately 0.4 ppm  $\text{NH}_3$ , clinical signs of erratic swimming, lying on their sides on the bottom of the tank, and varying degrees of anorexia were observed. The clinical signs decreased with time, and by day 55 the only clinical sign observed was erratic and frantic swimming. Appetite improved across study days and compared to that of control fish was considered to be fair or good. No mortalities were observed, but fish with clinical signs were removed for pathologic examination. On study day 60, fish from the tank receiving the approximately 0.4 ppm  $\text{NH}_3$  treatment had increased numbers of acidophilic droplets in the proximal tubular epithelial cells. Other studies have reported changes in gill epithelium. Most fish species are considered to be resistant to blood ammonia-induced neuropathy (Ip and Chew, 2010).

Studies have been done on the toxicity of  $\text{NH}_3$  in Atlantic salmon (*Salmo salar*) (Knoph and Thorud, 1996). In a subacute study, fish weighing  $369 \pm 70$  g were exposed to  $\text{NH}_3\text{-N} + \text{NH}_4\text{-N}$  to give TA-N at 0.5 (control), 3, 22, 40, 112, and 255 ppb and 0.05–25.3 ppm TA-N. The exposure was done in a running seawater system maintained at 8 or 9°C, 33–35‰ salinity, pH 7.8 or 7.9, and 6.3–9.7 ppm  $\text{O}_2$ . Parameters monitored were plasma TA; urea, glucose, osmolality ( $\text{OM}_p$ ), and ion levels; and the hematology parameters hematocrit (Hct), erythrocyte osmotic fragility (EOF), red blood cell numbers (RBCN), and the mean corpuscular volume (MCV). These parameters were measured after 2 or 3 and 14 or 15 days of exposure. It was found that plasma TA increased linearly with the water TA-N levels, and plasma urea generally decreased with increasing water TA-N levels, except for fish at the 25.3 ppm TA-N. At the 25.3 ppm TA-N level, there were large increases in  $\text{OM}_p$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Mg}^{+2}$ , urea, and TA levels, whereas glucose and  $\text{Ca}^{+2}$  were moderately increased and Hct decreased. All fish were dead by

day 6. At the 22 ppb TA-N exposure level, glucose,  $\text{OM}_p$ , and plasma ion levels and Hct, RBCN, EOF, and MCV were not affected at day 15. The authors concluded that the NOEC of 2.40 ppm TA-N should be safe for Atlantic salmon. Another study showed that cortisol levels were increased by exposure to approximately 13 ppm TA-N (Knoph and Olsen, 1994). The effect of 6 weeks of elevated ambient TA-N in Nile tilapia (*Oreochromis niloticus*) was studied by Benli *et al.* (2008). The TA-N concentrations were background, 1, 2, 5, and 10 ppm TA-N. Fish exposed to elevated TA-N had histopathologic lesions in the gills consisting of hyperemia, chloride cell proliferation, fusion of the secondary lamellae, and telangiectasis. Cloudy swelling and hydropic degeneration were observed in the liver, and hyperemia and glomerulonephritis were observed in the kidney.

### Nitrite

Nitrite ( $\text{NO}_2^-$ ) has a physiological endogenous existence in fish as an oxidate metabolite of the gaseous messenger nitric oxide (Jensen and Hansen, 2011). The presence of nitrite in ambient water can be toxic to aquatic animals (Camargo *et al.*, 2005). Aquatic animals, especially freshwater fish and crustacea, are more at risk for  $\text{NO}_2^-$  poisoning than are terrestrial organisms (Boyd, 1982; Boyd and Tucker, 1998; Camargo *et al.*, 2005). Nitrite values for human water safety generally do not apply to aquatic organisms. Nitrite intoxication of aquatic animals can occur when conditions exist for imbalance in the nitrogen cycle. Freshwater fish are more susceptible to  $\text{NO}_2^-$  than are saltwater fish. In freshwater fish,  $\text{NO}_2^-$  is rapidly absorbed across the gills. The generally accepted mechanism for  $\text{NO}_2^-$  absorption is by competing for  $\text{Cl}^-$  uptake across the gills. The chloride ion is exchanged for bicarbonate ( $\text{HCO}_3^-$ ), and this exchange occurs in the apical part of the gill epithelial cell. Nitrite has affinity for the  $\text{Cl}^-/\text{HCO}_3^-$  exchange protein and replaces  $\text{Cl}^-$  in the exchange process. Increasing the water concentration of Cl reduces the toxicity of  $\text{NO}_2^-$ . Fish with high  $\text{Cl}^-$  uptake, such as rainbow trout, perch (family Percidae), and Northern pike (*Esox lucius*), are more susceptible to  $\text{NO}_2^-$ . Millimolar  $\text{NO}_2^-$  levels in such fish, especially if  $\text{Cl}^-$  in the water is low, can result from micromolar levels of  $\text{NO}_2^-$  exposure from water. Nitrite causes a net loss of  $\text{Cl}^-$  because there is a reduction in the influx of  $\text{Cl}^-$ , and it stimulates a net loss of potassium ions from muscle, causing an extracellular hyperkalemia and increased excretion of potassium. Nitrite also enters red blood cells and oxidizes  $\text{Fe}^{+2}$  to  $\text{Fe}^{+3}$ , forming methemoglobin (metHb) (Grabda *et al.*, 1974). The metHb cannot carry oxygen, and oxygen tensions in blood approach the values for dissolved oxygen in the water. The oxidation of iron changes oxygen affinity of hemoglobin (Hb). Changes in oxygen affinity and blood

pH increase movement of oxygen to the swim bladder, thus affecting buoyancy. The stress response in teleost (bony) fish is  $\text{NO}_2^-$ -mediated disruption of the  $\text{Na}^+/\text{H}^+$  exchange mechanism in the red blood cell. This can further decrease the affinity of Hb for oxygen. Decreased oxygen tensions increase anaerobic metabolism and acidosis occurs. Exposure of rainbow trout to 1 mmol  $\text{NO}_2^-$  causes vasodilation and an increased cardiac workload (Aggergaard and Jensen, 2001; Jensen and Agnisola, 2005). The fish died when the metHg was greater than 70% and plasma  $\text{NO}_2^-$  level was 2.9 mmol at the time of death. Liver cell damage linked to mitochondrial pathology occurs, and hepatic stores of glycogen are depleted. Necrosis of the retina can occur. Hepatocytes from rainbow trout have been shown to detoxify  $\text{NO}_2^-$  by converting  $\text{NO}_2^-$  to considerably less toxic nitrate ( $\text{NO}_3^-$ ) (Doblender and Lackner, 1996). Recovery from nitrite poisoning appears to take several weeks, and compensatory gain may or may not occur.

Species susceptibility to  $\text{NO}_2^-$  is variable and is linked to  $\text{Cl}^-$  uptake by the gills (Durborow *et al.*, 1997; Jensen, 2003). Largemouth (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieu*), bluegill (*Lepomis macrochirus*), and green sunfish (*Lepomis cyanellus*) are resistant to high concentrations of  $\text{NO}_2^-$ . Catfish (order Siluriformes), goldfish (*Carassius auratus auratus*), fat-head minnows (*Pimephales promelas*), and tilapia (genus *Tilapia*) are sensitive to  $\text{NO}_2^-$ , whereas cold-water fish such as rainbow trout are highly sensitive to  $\text{NO}_2^-$ .

Nitrite poisoning (brown blood disease) can be prevented by adding  $\text{Cl}^-$  to the water (Durborow *et al.*, 1997). The most common option is to add  $\text{Cl}^-$  to the water to achieve a ratio of  $\text{Cl}^-$  to  $\text{NO}_2^-$  of 10:1. Decreased feeding rates and increased throughput of non-recycled water are alternative methods of controlling  $\text{NO}_2^-$ . Bacterial and parasitic disease increases the sensitivity of fish to  $\text{NO}_2^-$  poisoning. The presence of concurrent infectious disease requires increasing the concentration of ambient  $\text{Cl}^-$ . Catfish producers commonly maintain 100 ppm  $\text{Cl}^-$  in the pond or tank water as insurance against an increase in  $\text{NO}_2^-$  levels or to counteract the effects of concurrent infectious disease. Adult carp were exposed to 1 mM nitrite in ambient water for 48 h (Jensen *et al.*, 1987). Nitrite uptake and changes in blood respiratory properties, extracellular electrolyte composition, and acid-base status were examined. Nitrate in plasma increased to 5.4 mM within 48 h. Methemoglobin increased to 83% as plasma  $\text{NO}_2^{1-}$  increased, and arterial oxygen content decreased to very low values. Plasma  $\text{Cl}^{1-}$  decreased as plasma  $\text{NO}_2^{1-}$  increased.

### Nitrate

Chronic  $\text{NO}_3^-$  poisoning in rainbow trout was studied using 26.2 ppm  $\text{NO}_3^-$  and a static tank system (Grabda *et al.*, 1974). Fish were observed to gather around the

aeration system. Liver pathology was observed, and the hemopoietic centers were considered to be abnormal.

## Metals

### Copper

Copper (Cu) is an essential trace nutrient for fish. There are many sources of copper ions that can potentially enter aquaculture systems, including surface water. Runoff from lands receiving swine and poultry manure can be a source of Cu. Copper compounds are commonly used in aquaculture as an algicide and as a treatment for parasites. In freshwater aquatic systems, Cu exists in complexes with organic matter, complexed with other chemicals and weakly associated with water molecules (U.S. Environmental Protection Agency (USEPA), 2007). All of these factors can affect the bioavailability of Cu in ambient water. USEPA uses the biotic ligand model (BLM) to assess Cu toxicity. The model assumes that free Cu and Cu monohydroxide bind to a biotic ligand on the organism's surface. Death occurs when a critical amount of the total biotic ligand sites are attached to Cu. Water chemistry is used to predict the amount of Cu that will accumulate on/in the gill of a freshwater fish and estimates toxicity from a relationship between gill Cu accumulation and mortality (Grosell *et al.*, 2007). Multiple studies demonstrate that gill rapidly accumulates Cu following the onset of waterborne exposure, and the accumulation of Cu results in the disturbance of multiple physiological processes. The USEPA BLM does not consider differences in sensitivity due to size of the fish. There is evidence that variation in Cu toxicity between species is accounted for by differences in physiology.

The uptake of copper ions across the gill of fish is dependent on many parameters, including water hardness (levels of calcium and magnesium ions). There is generally a small margin of safety for many aquatic species for Cu ions. Cupric ions ( $\text{Cu}^{2+}$ ) disrupt the ATP-dependent sodium/potassium pump located in the gill chloride cells. This allows increased efflux of sodium ions. Cupric ions also replace calcium ions at the tight junctions, resulting in an efflux of sodium ions. The net loss of sodium ions results in disruption of osmoregulation and cardiovascular collapse. Copper ions are neurotoxic in fish, disrupting the olfactory and mechanosensory systems (Baldwin *et al.*, 2003; Linbo *et al.*, 2006). Peripheral olfactory function is inhibited at levels as low as 4  $\mu\text{g/L}$ , and loss of neuromasts occurs at concentrations greater than 20  $\mu\text{g/L}$ . Exposure to copper ions also decreases immune function. Copper levels can be difficult to interpret because pH, carbonate ions, and dissolved organic carbon are interactive in forming unavailable forms of copper, and  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$

compete for copper uptake by the fish (Sciera *et al.*, 2004). Changes in these parameters can cause a 60-fold difference in the lethal toxicity of Cu. Changes in water salinity at the extremes increase Cu toxicity in fish (Grosell *et al.*, 2007). Decreasing the pH increases the toxicity, and a 100 times increase in toxicity can occur with each unit decrease in pH. Warm-water fish are more tolerant of copper than are cold-water fish. In a study of fathead minnows, increasing sodium chloride and calcium chloride levels in the water decreased copper toxicity, whereas increasing potassium chloride increased sensitivity to Cu (Erickson *et al.*, 1996).

## Chlorine

Water is not considered safe for fish if a measurable level of chlorine ( $\text{Cl}_2$ ) is present. Morbidity can occur at 0.02ppm chlorine and mortality at 0.04ppm (Hadfield *et al.*, 2007). Municipal tap water can contain chlorine at 2ppm. Chlorine, chloramines, and other chlorine compounds are used as disinfection agents in municipal water and in aquaculture to disinfect ponds and tanks (Boyd and Tucker, 1998). Chlorine gas added to water forms a number of compounds (hypochlorous acid, hydrochloric acid, and hypochlorite), with the concentration of dissociated ion depending on the pH of the water. Chloramine is also formed by the reaction of chlorine with ammonia in water. Chlorine dioxide is used as a water disinfection agent and is reduced to chlorite. Chlorine dioxide is approximately 16 times more toxic to fish than is chlorite (Svecevicius *et al.*, 2005). For rainbow trout, safe levels for chlorine dioxide appear to be approximately 0.2ppm and approximately 3ppm for chlorite. The toxicity of chlorine residues is variable due to effects of water temperature. Residual chlorine in the water is generally oxidative and causes irritation and damage to the gills. The acute gill lesion is necrosis of gill epithelium occurring at higher chlorine concentrations, whereas the subacute and chronic lesions are gill epithelial hypertrophy and hyperplasia. Gill lesions not only cause hypoxia but also affect the acid-base homeostasis of fish. In this regard, gill damage in fish is akin to a mammal suffering concurrent pneumonia and nephritis.

## FEED

### Mycotoxins

#### *Fumonisin and moniliformin*

Fumonisin (FBs) are primarily produced by *Fusarium verticillioides*, *Fusarium proliferatum*, and 11 other *Fusarium*

spp. (Coppock and Jacobsen, 2009). Of the FBs,  $\text{FB}_1$  is considered to be the most toxic. Fumonisin is structurally similar to sphingosine, and they inhibit ceramide synthase, an enzyme in the sphingolipid synthesis pathway. Moniliformin (MON) is produced by *F. proliferatum* and other *Fusarium* spp. (Jestoi, 2008). MON inhibits pyruvate dehydrogenase and possibly other mitochondrial enzymes.  $\text{FB}_1$  and MON have been shown to reduce the productivity of fish. Nile tilapia (*O. niloticus*) fingerlings were fed  $\text{FB}_1$  or MON at 0, 10, 40, 70, and 150ppm for 8 weeks (Tuan *et al.*, 2003). The  $\text{FB}_1$  and MON were extracted from cultures of *Fusarium moniliforme* and *F. proliferatum*, respectively. Mortalities in all treatment groups were low and were not dose related. Feeding diets containing 150ppm  $\text{FB}_1$  or MON decreased hematocrit. Both mycotoxins disrupted metabolism. There was evidence that sphingolipid metabolism was disrupted in fish fed  $\text{FB}_1$ . Feeding MON increased serum pyruvate. For fish fed MON, decreased weight gains were observed at the 70 and 150ppm levels, and for fish fed  $\text{FB}_1$ , decreased weight gains were observed at the 40, 70, and 150ppm levels. Fish fed 10ppm  $\text{FB}_1$  had decreased weight gains for the first 2 weeks, but body weights at 4 weeks were not significantly different from those of controls. Histopathology linked to treatments was not observed in the fish. Studies on the combination of  $\text{FB}_1$  and MON showed interactions between the two toxins (Yildirim *et al.*, 2000). Channel catfish (*Ictalurus punctatus*) are more sensitive to  $\text{FB}_1$  and MON than are tilapia, and  $\text{FB}_1$  is more toxic than MON in channel catfish. Yearling carp were fed rations containing 10 and 100ppm of added fumonisin  $\text{B}_1$  for 42 days (Petrinec *et al.*, 2004). Observed histopathology included increased rodlet cell numbers in and around damaged tissues, with many damaged blood vessels containing stacks of rodlet cells outside the endothelium. Scattered lesions were observed in the exocrine and endocrine pancreas and interrenal tissues. The subchronic toxicity of  $\text{FB}_1$  was studied in 120–140g carp (*Cyprinus carpio* L.) (Pepeljnjak *et al.*, 2003). A balanced fish diet provided a dose of 0.5 and 5.0mg  $\text{FB}_1$ /kg body weight for 42 days. Fish on the 5mg  $\text{FB}_1$ /kg dose lost body mass and had a higher incidence of erythrodermatitis cyprini (*Aeromonas salmonicida* subsp. *nova*). Treatment with  $\text{FB}_1$  increased erythrocyte and platelet numbers. Also increased were serum levels of creatinine, total bilirubin, and the activities of aspartate aminotransferase and alanine aminotransferase. The  $\text{FB}_1$  diets were not lethal to the fish, but the parameters studied indicate the kidney and liver as target organs.

#### *Aflatoxins*

Fish feeds can be contaminated with aflatoxins (AFs). Aflatoxins were identified in the early 1960s as causing hepatic cancer in fish (Sinnhuber and Wales, 1978).



Rainbow trout are sensitive to AFs, and dietary levels as low as 0.4ppb fed for less than 1 year cause hepatic cancer. Salmon (family Salmonidae) are more resistant than rainbow trout to the carcinogenic effects of AFs. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is metabolized by the P450 enzyme system in the liver of fish to form the AFB<sub>1</sub>-8,9-epoxide. The AFB<sub>1</sub>-8,9-epoxide forms adducts with DNA, RNA, and proteins. The formation of adducts is considered to be the primary mechanism of action for the carcinogenicity of AFB<sub>1</sub>. *In vitro* cultures of rainbow trout peripheral blood leukocytes incubated with AFB<sub>1</sub> showed the trout leukocytes to be 1000 times more sensitive than mouse leukocytes to AFB<sub>1</sub> (Ottinger and Kaattari, 1998). Exposure of trout embryos to AFB<sub>1</sub> causes long-term immunodysfunction (Ottinger and Kaattari, 2000). Fry are considered to be more sensitive than mature fish to AFs. Feeding a diet containing 500ppm AFB<sub>1</sub> altered immune parameters and decreased the native resistance to disease in India major carp (*Labeo rohita*) (Sahoo and Mukherjee, 2003). AFB<sub>1</sub> has been studied in tilapia (Nguyen Anh *et al.*, 2002). One-month-old tilapia fingerlings were fed diets containing 0, 0.25, 2.5, 10, or 100ppm AFB<sub>1</sub> for 8 weeks. Fish fed 10 and 100ppm AFB<sub>1</sub> consumed less feed and expelled the feed after ingestion, suggesting feed refusal. At 6 weeks of feeding, the survival rate for the fish fed the 100ppm level of AFB<sub>1</sub> was 55%. Weight gains for the fish fed the diets containing 2.5ppm AFB<sub>1</sub> or greater were decreased. Hematocrit was reduced in the fish fed diets 0.25ppm AFB<sub>1</sub> or greater. Fish fed diets containing 10 and 100ppm AFB<sub>1</sub> had liver lesions. Pleiomorphic liver nuclei were present in 86% of the fish fed the diet containing 10ppm AFB<sub>1</sub> and 100% of the fish fed the diet containing 100ppm AFB<sub>1</sub>. Catfish have a high resistance to the carcinogenic effects of AFB<sub>1</sub>, and this is linked to a low rate of formation of the AFB<sub>1</sub>-8,9-epoxide (Gallagher and Eaton, 1995). Dietary vitamin C was shown to provide protection against AFB<sub>1</sub>.

There are concerns that dietary AFs can cause residues of AFs in edible fish tissues. The toxicokinetics of AFs were studied in rainbow trout in a 21-day study (Nomura *et al.*, 2011). A rainbow trout diet had pure AFs added to give various levels of AFs as AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub>. The uptake factor for muscle was calculated by dividing the level of AF in muscle by the AF level in feed (AF muscle/AF feed). The uptake factors were  $0.70 \pm 0.08 \times 10^3$ ,  $0.48 \pm 0.04 \times 10^3$ ,  $0.39 \pm 0.08 \times 10^3$ , and  $0.67 \pm 0.08 \times 10^3$ , respectively, for AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, and AFG<sub>2</sub>. The levels of AFB<sub>1</sub> in liver were 165–342 times higher than those in muscle. Aflatoxicol (AFL) was identified as a major metabolite of AFB<sub>1</sub>, and in muscle the AFL levels were two orders of magnitude higher than those of AFM<sub>1</sub>. AFL and AFM<sub>1</sub> were detected in muscle and liver and also in the rearing water. The elimination rate constant ( $\alpha$ ) of AFB<sub>1</sub> was 1.83 and 1.38 per day for muscle and liver, respectively. The  $\alpha$  value for AFL in muscle was 2.02 per day, and that for liver was 2.41 per day.

The half-life ( $t_{1/2}$ ) of AFB<sub>1</sub> in muscle was approximately 9.1h, and the  $t_{1/2}$  in liver was 12.0h. The  $t_{1/2}$  of AFL was approximately 8.2h and approximately 7.0h in muscle and liver, respectively. This study shows that for fish consuming a diet containing approximately 500ppb and switched to a diet that does not contain AFB<sub>1</sub>, approximately 37h is required for AFB<sub>1</sub> to be cleared (<20ng AFB<sub>1</sub>/kg of muscle) from muscle tissue. This study also shows that AFs are not biomagnified in fish.

### Ochratoxins

Ochratoxins A (OTA), OTB, and OTC are primarily produced by *Aspergillus alutaceus* var. *alutaceus* (syn. *Aspergillus ochraceus*), *Penicillium verrucosum* (Dierckx), and *Penicillium verrucosum viridicatum* (Westling) (Jacobsen *et al.*, 2007; Coppock and Jacobsen, 2009). Other *Aspergillus* spp. and *Penicillium* spp. have been reported to produce ochratoxins. *Penicillium* species are the most important in temperate climates and *A. alutaceus* var. *alutaceus* in tropical climates. All of these fungi grow under storage conditions when in equilibrium with 80–85% moisture (~16 to 18% for starchy cereal grains) and temperature values as low as 10°C. Early studies of parenteral exposure of rainbow trout to ochratoxins showed that OTA was approximately 10 times more toxic than OTB. OTA extracted from cultures of *A. ochraceus* was added to channel catfish diets at 0, 0.5, 1.0, 2.0, 4.0, or 8.0ppm (Manning *et al.*, 2003). The average initial weight was 6g/fish. Decreased body weight gain was observed at 2 weeks and at 8 weeks in the catfish fed the diet containing 1.0ppm OTA. At 8 weeks, a decrease in body weight gain was observed for the catfish fed the 2, 4, and 8ppm diets. Hematocrit was decreased in the fish fed the 8ppm diet, and white blood cell numbers were not changed. At 8 weeks, there was 80% survival in the fish fed the diet containing 8ppm OTA. Histopathologic lesions were observed in fish fed the OTA diet at the 1, 2, 4, and 8ppm levels. The lesions were described as enlarged melanomacrophage centers replacing the hepatopancreatic cells and the presence of melanomacrophage centers in the posterior kidney. These findings suggest that the hepatopancreatic cells and the kidney are targets for OTA in catfish. Diets containing 400 and 600ppb ochratoxin were fed to Nile tilapia (*O. niloticus*) with approximately 55-g body weight (Shalaby, 2004). Ochratoxin caused a significant decrease in red blood cell numbers, Hb, and Hct. The activity of alanine aminotransferase in liver and muscle was increased.

## Contaminants in aquaculture fish

### Chlorinated dioxins

Aquaculture animals are a dietary source of polychlorinated dibenzodioxins (PCDDs), polychlorinated



dibenzofurans (PCDFs), and polychlorinated biphenyl (PCBs) compounds. Each of these groups is a mixture of compounds. These groups of compounds are collectively referred to as persistent organochlorines (POCs). Health concerns exist because these compounds are bioconcentrated in body fat of animals. These compounds are also transferred to the human fetus during pregnancy and excreted in breast milk during lactation. Dietary animal protein, fat and clays are the predominant source of the POCs for cultured fish. Catfish have been identified as an important source of PCDDs for persons consuming them, and in these individuals, this can exceed the dietary exposure from dairy products (Jensen and Bolger, 2001). In a study on the transfer of PCDDs and PCDFs from diet to muscle fat, it was found that in the first 13.5 months of feeding, approximately 30% of the dietary PCDDs and PCDFs in a diet fed to rainbow trout were transferred to fat located in muscle tissue (Karl *et al.*, 2003). At 19 months, the transfer rate increased slightly to approximately 34% for male trout and decreased to approximately 27% for egg-laying female trout. A congener-specific pattern of PCDDs and PCDFs accumulation in the fish was not identified. The concentrations of PCDDs and PCDFs in diet were directly related to the concentrations of PCDDs and PCDFs in muscle fat. The levels of PCDDs and PCDFs in muscle fat increased during the 19-month feeding period.

### Methylmercury

Methylmercury (meHg) is a concern with regard to human foods from aquatic sources. The primary source of meHg in aquaculture is the use of fish byproducts in feedstuffs. Methylmercury is formed by biota in the benthic region of the aquatic system, and it is biomagnified in the food web. In fish, meHg has affinity for skeletal muscle and is bioaccumulated in skeletal muscle (Berntssen *et al.*, 2004). Approximately 23% of the dietary meHg and approximately 6% of dietary inorganic mercury (Hg) are absorbed by fish. Fish fed diets containing 5 and 10 ppm meHg for 4 months had 1.1 and 3.1 ppm of meHg in muscle (freeze-dried), respectively. The threshold toxic level for Atlantic salmon is estimated at 0.5 ppm meHg in diet. Studies in fathead minnows showed that the threshold level of dietary meHg on spawning parameters is less than 0.88 ppm (Hammerschmidt *et al.*, 2002).

## TOXICOLOGY INVESTIGATIONS

The triggers for a toxicological investigation generally are either increased mortality or decreased performance in reproduction, feed conversion, and growth. Food safety issues can also be another trigger. It is

important to document both when the toxicological incident occurred and any concurrent environmental events, changes in management, etc. Review of records can be helpful to determine when the incident started, and records/printouts from equipment monitoring water parameters are very important in this process. A complete history is important to direct the diagnostic procedures and analytical toxicology. The following are important questions to ask:

- Was the incident an acute event or did it occur over a period of time?
- What are the size, age, and location of the fish that are affected?
- Were clinical signs observed?
- Can the onset be linked to changes in feed sources, shipments of feed or feed ingredients, or a particular feed storage area?
- Are there links to changes in water handling, filtering, interruptions in electrical power, or a rainfall event?
- Are there links to ambient water temperature?
- Pesticide use and location of use?
- Use of drugs?
- Did deaths or disease also occur in terrestrial animals?
- Is the death of aquatic animals linked to a die-off of aquatic plants and phytoplankton?
- Previous land use in the area where the ponds have been constructed?
- Are pathology, analytical toxicology and feed ingredients data available?

The time of day that mortalities occur is important. These can be linked to changes in water temperature and a decrease in dissolved oxygen. Sporadic mortality is more likely to be related to parasites or changes within the tank and pond system. As a general rule, small fish are more resistant to low dissolved oxygen and are less resistant to chemical substances.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Toxic substances and toxins generally enter fish aquaculture systems via the feed, from runoff or other liquid contamination of the water in which the fish are kept, or develop from the chemical dynamics of substances that are naturally in the water column. Fish may exacerbate the latter by their excretion products and sheer biomass in aquaculture systems. The toxicological security of fish stocks requires effective quality assurance and quality control programs in two main areas. Manufacturers and suppliers of feedstuffs to the aquaculture industry

can play a major role by ensuring that toxic substances and toxins do not enter the food chain. The operators of fish culture facilities control the aquatic environment, at least for pond- and tank-reared fish, and therefore have the main role in monitoring water for levels of toxic substances and ensuring that natural drainage of surface water does not introduce toxic substances into the aquaculture system.

Multiple uses of increasingly limited water resources present challenges of access to the aquaculture industry. Securing the quality of incoming water supplies in the face of multiple other uses for water and requirements to return the water to the environment in pristine condition will be ongoing concerns for the industry. High capital investment and operating costs also constitute constant challenges. Increased use of and research into recirculating systems will pose significant future research challenges.

The culture of aquatic organisms for food, environmental reconstitution, and a variety of other purposes becomes more common and more important year by year. This trend will continue into the foreseeable future. Toxicity due to factors inherent to the culture of large biomasses of fish in relatively small volumes of water or introduced through feed or water poses a threat not only to the fish but also to the humans or animals that consume those fish or fish products. Dietary adequacy and feed safety can be issues in aquaculture. Food safety related to fish products will become a greater societal issue in the future.

An emerging area of aquatic toxicology will be related to complex systems using fish, other aquatic organisms, and plant communities to clean sewage, waste, and other degraded water sources in environmental restoration systems. Such future toxicological challenges will not only include disturbances of well-known water parameters as described previously but also likely extend to complex sulfur/sulfate/sulfide toxicities and organic acid toxicities, among others.

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# Toxicology and diversity of marine toxins

Aurelia Tubaro, Silvio Sosa and James Hungerford

## INTRODUCTION

Marine toxins are a structurally and toxicologically diverse group of compounds with potent biological activity. Like the mycotoxins, marine toxins are non-proteinaceous, are heat stable, and many are smaller than 1000 Da. With the exception of the tetrodotoxins with their bacterial origins, marine toxins are secondary metabolites of microalgae and often referred to as phycotoxins. The role of these secondary metabolites is not completely clear, but chemical defense is among their prominent hypothesized functions. The unicellular algae producing the toxins are themselves a diverse group, ranging from dinoflagellates to diatoms and even cyanobacteria. Dinoflagellates produce the majority of marine toxins. Remarkably, these microorganisms are unusual in having large genomes, some of which are 100 times larger than the human genome (Lin, 2006), which complicates use of modern genetic approaches in some marine toxin biosynthesis studies (Kalaitzis *et al.*, 2010). Only since 1987 have diatoms been known to produce marine toxins, and even then, production is restricted to the genus *Pseudonitzschia*. Marine cyanotoxins show considerable diversity (Golubic *et al.*, 2010), and some are neurotoxic (Araoz *et al.*, 2010). The vast majority of cyanotoxin-implicated poisonings involve terrestrial cyanobacteria, which are discussed elsewhere in this book.

Most, but not all, of the marine toxins exert their toxic effects by disrupting the nervous system. Other marine toxins impact protein phosphorylation or other critical processes. Their relative toxicities also vary considerably. Among the neurotoxins, there is again considerable diversity, with many of them binding to the

voltage-gated sodium channel (VGSC) and others binding to excitatory amino acid receptors and disrupting synaptic transmission. By far, those causing the greatest numbers of illnesses and some deaths share the common trait of binding to receptors of the VGSC to disrupt neurological function.

The impact of marine toxins on marine wildlife and humans is significant. Marine toxins are responsible for avian poisonings; massive fish kills; birth morbidity and mortality; and sanitary, ecological, and economic problems. Marine mammals are also impacted by marine toxins, including many fatalities, and an important and global ecological role for marine toxins in their health and survival has been suggested (Trainer and Baden, 1999). Most of the known marine toxins are a threat to human health and, especially among the marine neurotoxins, some can be fatal (Van Dolah, 2000). However, the marine toxins discussed here are not venoms: they are accumulated from the environment rather than being produced by a specialized toxin-producing gland. Poisonings of humans and other animals result from consumption of contaminated food at some level of marine food webs (Isbister and Kiernan, 2005). Although certain marine toxins can contaminate finfish, predominantly it is molluscan shellfish, due to their filter feeding on toxic algae, that are the target of the most extensive monitoring efforts. In the United States, this includes coastal harvest areas managed by the shellfish-producing states (Hungerford and Wekell, 1993) and offshore federal resources (Etheridge, 2010). In Europe, there are many shellfish-producing countries, with the Galicia region (Spain) comprising the greatest percentage among producers, and methodologies and regulation are the realm of the EU Reference Laboratory on Marine



Biotoxins and the European Community's DG-SANCO, respectively. Shellfish contaminated by algal toxins, when not properly monitored, can cause severe and life-threatening human poisonings. Shellfish poisonings account for 7.4% of marine intoxications in the United States (Isbister and Kiernan, 2005). All of these reported shellfish poisonings were due to recreational harvest, underscoring the effectiveness of monitoring and the safety of commercial shellfish product in the United States.

Regardless of the specific mechanism of toxicity, many of the marine toxins present the same limitations and challenges in both toxicological studies and in their management. Specifically, it is the very limited availability, particularly of large quantities of highly purified toxins needed for toxicological studies that has hampered and sometimes halted many research efforts addressing these and other algal toxins. Indeed, many of the toxins are not commercially available in any quantity. In many toxicological studies, a reduced number of animals are used, and sometimes, to maximize the toxicological effects of the test material, parenteral routes of administration are used. On the contrary, very few studies have examined the effects after oral intake, the usual exposure route. This chapter focuses on the toxicology of the most dangerous marine toxins for humans. The major clinical poisonings due to the consumption of fish and shellfish are also covered.

For the past few decades, detailed structural knowledge of the marine toxins, once obscured by the use of mouse bioassays in their detection, has been growing rapidly. Much of the recent progress seen in marine toxin research concerns the multiplicity of toxin profiles within each toxin class. Due to increasing use of chemical detection methods, variations in marine toxin profiles, regional variations among toxic algae, and variations between vector species such as fish and shellfish have been known since the 1980s. Increasing use of liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis and certain *in vitro* tools, as well as improving commercial availability of a greater variety of toxin congeners, has greatly expanded our knowledge of the toxins. Extensive metabolism and other biotransformations of the marine toxins as they pass through various trophic levels are becoming a familiar theme as a result of these new studies. Most important, a more detailed understanding of the fate of the toxins is in turn supporting a more informed approach to updating detection methods used in monitoring. Considerable progress is now being made in the development, validation, and application of new methods for use in the monitoring of seafood contamination by marine toxins. This is resulting in the gradual replacement of outdated and unspecific mouse bioassays with modern analytical methods. These advances, along with the discovery of

new metabolites (and appreciation for their importance in our understanding and management of the toxins), are described in this chapter.

## SAXITOXINS

Saxitoxins (STXs), a family of hydrophilic toxins based on the parent STX, were discovered in the past century and can contaminate seafood and drinking water. The name of this toxin group is derived from *Saxidomus giganteus*, the clam from which it was purified for the first time. The ingestion of STXs-contaminated seafood provokes a paralytic syndrome known as paralytic shellfish poisoning (PSP), one of the most common and dangerous forms of shellfish poisoning. Although PSP cannot be classified as a major public health problem due to its relatively low incidence rate, it is of considerable concern because a fatal dose for humans can be obtained eating a single highly contaminated shellfish. There is no available antidote, although supportive treatment, if done promptly, is generally sufficient for this potent but reversibly binding group of toxins. Furthermore, STX is the only marine natural product that is a declared chemical weapon (Llewellyn, 2006).

### Background

Victims of PSP can develop symptoms within 5–30 min after contaminated shellfish consumption. The first effect is typically paraesthesia, with burning or tingling of the tongue and lips, which spreads to the face, neck, fingers, and toes. In more severe cases, this is followed by a feeling of numbness in fingertips and toes, which progresses to the arms, legs, and neck within 4–6 h. Death is usually caused by respiratory paralysis, and without mechanical respiratory support, the fatality rate is 5–10%. There is a good prognosis for individuals surviving beyond 12 h, although muscular weakness can persist following recovery. Other minor anecdotal symptoms reported are a feeling of dizziness or “floating” (due to distortion of sensation and proprioception), generalized paraesthesia, arm and leg weakness, and ataxia. Headache, nausea, and vomiting can also occur in the initial phase of the poisoning. Reflexes may be normal or absent, and patients may remain conscious and alert throughout the poisoning. In patients with mild to moderate poisoning, the symptoms resolve in 2 or 3 days, but in more severe cases, weakness may persist for 1 week (Isbister and Kiernan, 2005; Llewellyn, 2006).

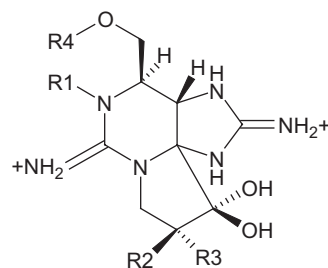
PSP diagnosis is based on characteristic neurological clinical signs and on a history of recent ingestion of

seafood (most often bivalve shellfish, hence the term PSP). The symptoms of PSP are difficult to differentiate from those of tetrodotoxin poisoning, due to the (pharmacological) similarities of the toxins.

The toxins responsible for PSP, STX, and its derivatives, including gonyautoxins (GTXs), are water-soluble, heat-stable, tetrahydropurine compounds that are among the most potent known neurotoxins. The chemical structure of STX is represented by two guanidinium moieties joined in a stable azaketall linkage (Figure 69.1). More than 30 natural STX analogs, which vary mainly by different combinations of hydroxyl and sulfate substitutions at four sites on the molecule as well as by forming epimeric pairs via ring opening and closure, have been isolated to date. STXs can be divided into four groups based on substitution at R<sub>4</sub>: the carbamate toxins, the sulfocarbamoyl toxins, the decarbamoyl toxins, and the deoxydecarbamoyl toxins. The guanidinium group at positions C-7, C-8, and C-9 and the hydroxyl group at C-12 are essential for binding of STXs to Na<sup>+</sup> channels; the substitution at R<sub>4</sub> leads to substantial changes in toxicity, with the carbamate toxins being the most potent group (Ciminiello and Fattorusso, 2004; Isbister and Kiernan, 2005).

The toxins are produced by marine dinoflagellates, including *Alexandrium* ssp., *Pyrodinium bahamense* var. *compressum*, and *Gymnodinium catenatum*. Shellfish accumulate the toxins by filter feeding the toxic microalgae. PSP toxins have also been identified in freshwater blue-green algae (cyanobacteria) species, but no human poisoning has been reported. Also, red algae of the genus *Jania* were reported to produce STX. Controversial is the production of STXs by a symbiotic bacteria isolated from *Alexandrium tamarense* (Isbister and Kiernan, 2005; Llewellyn, 2006).

Shellfish contamination by STXs is a global concern, being endemic in northwest America. PSP cases have also occurred in Chile, Taiwan, Japan, South Africa, Australia, India, England, Guatemala, Costa Rica, Singapore, Canada, Spain, and Mexico (Gessner, 2000; Llewellyn, 2006). STXs are concentrated by bivalve shellfish such as clams, oysters, mussels, and scallops but also by crabs, puffer fish, gastropods, and cephalopods. Shellfish contamination can persist for a long time after exposure to the toxic microalgae, although in most cases, bivalves clear the toxin quickly enough to allow later reopening of harvest areas. Although PSP is widely distributed, crustacean-induced PSP seems to be concentrated in the tropics, where predator crabs of the Xanthidae family are often contaminated by paralytic toxins and have caused human deaths. In recent years in Florida, puffer fish thought to be *Sphoeroides nephelus* provoked the poisoning of more than 12 people due to the presence of large amounts of PSP toxins (Llewellyn, 2006). The occurrence of STX as the major toxin in



Toxin	R1	R2	R3	R4
STX	H	H	H	
neoSTX	OH	H	H	
GTX1	OH	H	OSO <sub>3</sub> <sup>-</sup>	H <sub>2</sub> N-CO
GTX2	H	H	OSO <sub>3</sub> <sup>-</sup>	
GTX3	H	OSO <sub>3</sub> <sup>-</sup>	H	
GTX4	OH	OSO <sub>3</sub> <sup>-</sup>	H	
dcSTX	H	H	H	
dcNEO	OH	H	H	
dcGTX1	OH	H	OSO <sub>3</sub> <sup>-</sup>	H
dcGTX2	H	H	OSO <sub>3</sub> <sup>-</sup>	
dcGTX3	H	OSO <sub>3</sub> <sup>-</sup>	H	
dcGTX4	OH	OSO <sub>3</sub> <sup>-</sup>	H	
B1 (=GTX5)	H	H	H	
B2 (=GTX6)	OH	H	H	
C3	OH	H	OSO <sub>3</sub> <sup>-</sup>	-O <sub>3</sub> S-NH-COO
C1	H	H	OSO <sub>3</sub> <sup>-</sup>	
C2	H	OSO <sub>3</sub> <sup>-</sup>	H	
C4	OH	OSO <sub>3</sub> <sup>-</sup>	H	

FIGURE 69.1 Chemical structure of saxitoxin and some of its analogs (from Luckas *et al.*, 2003).

another puffer fish (*Arothron firmamentum*) has also been reported (Nakashima *et al.*, 2004).

The STXs have also been implicated in the deaths of humpback whales feeding on STX-laden mackerel, with the further suggestion that the whales' diving adaptation and other physiological factors increase their susceptibility to STXs (Geraci *et al.*, 1989). STX can also be taken in directly through the gills of Atlantic salmon (Bakke and Horsberg, 2010), and the elimination half-life following intraperitoneal (i.p.) injection is 102 min. Monk seal mortalities have also been blamed on exposure to the STXs (Reyero *et al.*, 1999).

### Pharmacokinetics/toxicokinetics

Although the mechanism of action of STXs is well established at the molecular level, little is known about their absorption, distribution, biotransformation, and

elimination. The charged nature of STXs makes these molecules poor candidates for oral availability, but their uptake from the gut occurs within minutes from ingestion, and no toxin is eliminated in the feces of test animals (Llewellyn, 2006). Studies in animals and evidence from PSP poisonings in humans revealed that STXs are eliminated mainly through the urine (Stafford and Hines, 1995; Gessner *et al.*, 1997; Andrinolo *et al.*, 1999, 2002). The half-life of urinary elimination of a sublethal dose of STX in rats (2 µg/kg, i.v.) is approximately 90 min (Stafford and Hines, 1995). Urinary elimination of STX was also demonstrated in cats: after i.v. injection of 2.7 or 10 µg/kg STX, excretion involved glomerular filtration, which dropped to low levels at the highest dose due to STX hypotensive effect. The study also showed the presence of the toxin in liver, spleen, and brain (Andrinolo *et al.*, 1999). By oral route in cats, GTX 2/3 epimers (70 µg/kg) were completely absorbed at the intestinal level and, similarly to STX, the excretion involved glomerular filtration (Andrinolo *et al.*, 2002).

Metabolic transformation of paralytic shellfish toxins by mammals has been little studied. Serum and urine of poisoned humans revealed the occurrence of GTX2 sulfation, which is in contrast with the findings in rats, in which no metabolism of STX occurs during passage in urine. In another case report, STX was found mainly in the gut contents, whereas the urine contained only 50% STX, with neoSTX and dcSTX becoming quite prominent (Llewellyn, 2006). The marked difference between the toxin compositions in the ingested mussels and in human sera, as well as the similar toxin profile between sera and urine samples, suggests that PSP toxins are metabolized at gastrointestinal and/or hepatic levels (Gessner *et al.*, 1997). It has to be considered that the transformations of the PSP toxins in mammals can be the consequence of both biochemical metabolism and the conditions to which the toxins are exposed, such as the gastric juice, which can convert small amounts of the less toxic sulfated PSP toxins to more potent compounds (Llewellyn, 2006). An early study on clearance of PSP toxins from human serum showed completion in less than 24 h (Gessner *et al.*, 1997), and in 2007 detailed clearance data were obtained (Etheridge *et al.*, 2007) following a human outbreak of PSP: Rapid elimination from blood serum was observed, and urinary clearance of the toxins gave half-lives dependent on the toxin class, with gonyautoxins clearing three times faster than saxitoxin or neosaxitoxin.

## Mechanism of action

STX is a potent neurotoxin that selectively blocks the sodium channel by binding to site 1 (Cestèle and Catterall, 2000), preventing the entry of Na<sup>+</sup> ions into

cells, thus preventing nerve conduction and resulting in motor and sensory nerve abnormalities. All the STX analogs bind to the same (site 1) receptor, although with different affinities (Isbister and Kiernan, 2005). This binding site is shared with another group of sodium channel-blocking toxins, the tetrodotoxins. The VGSC binding sites for these and other marine toxins, and other natural toxins, were mapped during studies of the ion channels (Cestèle and Catterall, 2000).

## Toxicity

### Human toxicity

STXs provoke potentially lethal PSP in humans. The most common symptom of PSP, occurring in almost all affected, is perioral paraesthesias, described as either numbness or tingling. Among those displaying more severe illness, in some cases gastrointestinal symptoms (nausea, vomiting, abdominal pain, and diarrhea) were reported. More severe intoxication leads to a variety of neurological symptoms, including weakness, dysarthria, diplopia, ataxia, and vertigo or dizziness, and, in some cases, respiratory arrest or death. It is unclear whether PSP provokes significant direct effects on the myocardium (Lagos and Andrinolo, 2000). Both diastolic and systolic hypertension were reported in almost all the 11 PSP intoxicated patients studied by Gessner *et al.* (1997). Another report referred hypertension in 3 PSP patients from England. Experimental studies in cats and dogs emphasized a hypotensive effect after i.v. exposure to high doses of STX and a hypertensive effect at low doses (Lagos and Andrinolo, 2000). Death results from respiratory arrest and collapse, the terminal symptoms in patients without medical care. No long-term clinical effects of PSP have been reported (Gessner, 2000).

Several PSP incidents have been recorded throughout the world. The exposure to STX was estimated based on an examination of approximately 20 PSP incidents that occurred in Canada between 1970 and 1990 involving approximately 60 people (ages 3–72 years). The symptoms were classified as mild, moderately severe, or extremely severe. Mild cases were generally associated with an STX exposure of 2–30 µg/kg, whereas in the more severe cases STX exposure was greater than 10–300 mg/kg. Based on these data, a lowest-observable-adverse-effect level (LOAEL) of 2 µg/kg was established (FAO/IOC/WHO, 2005).

### Experimental toxicity

#### Single administration

The mouse LD<sub>50</sub> (median lethal dose) values of STX by i.v., i.p., and oral routes are 3.4, 10, and 263 µg/kg,

respectively. Significant species differences in oral toxicity have not been observed. The potency of different STXs varies widely. After i.p. injection in mice, the carbamates and the decarbamoyl derivatives are the most toxic PSP toxins, whereas the sulfocarbamoyl derivatives are the least toxic (Andrinolo *et al.*, 1999; Llewellyn, 2006).

## Treatment

Treatment for PSP is only supportive. Admission to an intensive care unit is necessary in moderate to severe cases to prevent respiratory failure: artificial respiration must be prompt and is the most vital treatment in many cases. Patients have to be carefully observed in the early stages of poisoning to immediately recognize and treat the progressing paralysis and respiratory failure.

## Concluding remarks

PSP due to the intake of toxins from the STXs group has provoked many fatalities. Because a specific antidote is not available for PSP, prevention is important and monitoring programs are carried out in many countries. A regulatory level of 0.8mg STX equivalents/kg shellfish meat has been set up in North America, in the European Community, as well as in many other countries worldwide. The AOAC International validated mouse bioassay (MBA) has been widely used in monitoring programs and has provided health protection (FAO/IOC/WHO, 2005). Three mice are intraperitoneally injected with the contaminated shellfish extracts, and their survival times are compared with those of mice injected with different concentrations of STX. The detection limit is only 0.4mg STX equivalents/g shellfish meat (Luckas, 1992). Various other methods, including cytotoxicity assays, receptor binding assays, immunoassay, surface plasmon resonance, and several chromatographic methods, have been developed to detect the paralytic toxins, as reviewed by Degrasse *et al.* (2010). AOAC-validated methods include the Lawrence pre-column liquid chromatography fluorescence method AOAC 2005.06 (Lawrence *et al.*, 2005) and the post-column oxidation method of CFIA (Rourke *et al.*, 2008; van de Riet *et al.*, 2011), which is officially approved as AOAC 2011.02. Both of these LC fluorescence methods can provide nearly full coverage of STXs (with the exception of GTX6 and dcNEO) and complete coverage of the most toxic forms. In Europe, method 2005.06 was approved in 2006, with some minor restrictions, as an alternative method to the MBA in monitoring programs. Also, as of 2010, in the United Kingdom, monitoring of the STXs is accomplished solely using a modified and

semi-automated version of this method (Turner *et al.*, 2009). Ireland and New Zealand made the same transition in 2011. Official AOAC approval of the post-column oxidation LC method AOAC 2011.02 for the saxitoxins will likely allow a phasing out of the mouse bioassay in Canada. Degrasse *et al.* (2010) compared performance and practical aspects of these LC fluorescence methods for the STXs. The receptor binding assay for paralytic shellfish toxins was interlaboratory studied (Van Dolah *et al.*, 2012) and approved as official method 2011.27 by AOAC. Official method status will increase the likelihood that one of these methods will be considered as a potential “type II” (reference) method in international application by Codex Alimentarius.

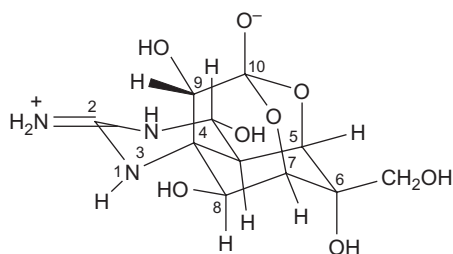
## TETRODOTOXIN

### Background

Puffer fish are an esteemed delicacy in Japan, despite the presence of a lethal toxin, tetrodotoxin (TTX), in the gonad, liver, viscera, and skin. TTX poisoning is the most common lethal marine poisoning. Its clinical effects develop rapidly: early symptoms are sensory, including perioral and distal numbness and paraesthesia. In mild poisoning cases, only sensory symptoms associated with minor gastrointestinal effects (mainly nausea and vomiting) occur. In moderately severe poisonings, patients develop distal muscle weakness and facial weakness and, later, ataxia and incoordination with normal reflexes. Dizziness can also be accompanied by a sensation of floating. Severe poisoning provokes generalized flaccid paralysis, respiratory failure, and aphonia. In severe and life-threatening poisoning, cardiovascular effects (bradycardia, hypotension, and dysrhythmias) occur together with respiratory failure and coma. Patients with severe poisoning rapidly progress to flaccid paralysis. Most moderate and severe cases generally resolve after 5 days or longer in severe poisoning. In minor cases, complete recovery occurs within a few hours (Isbister and Kiernan, 2005).

The symptoms are very similar to those of PSP due to the similar mechanism of action of the involved toxins. The anamnesis (if possible) and the analysis of uneaten fish or patient's urine or serum confirms diagnosis for tetrodotoxin. TTX and its analogs are water-soluble and heat-stable heterocyclic compounds with a guanidinium group that is positively charged in the biological pH range (Figure 69.2) (Yotsu-Yamashita *et al.*, 2003); they are not produced by microalgae but, rather, by other microorganisms. A bacterium initially classified as *Pseudomonas* sp. and later amended as *Shewanella alga* was confirmed to produce TTX (Yasumoto, 2000). A list





**FIGURE 69.2** Chemical structure of tetrodotoxin (from Yasumoto, 2000).

of bacteria known to produce TTX is reported by Yu *et al.* (2004). Yu *et al.* (2004) and Wu *et al.* (2005) referred other species of TTX-producing bacteria: *Microbacterium arabinogalactanolyticum*, *Serratia marcescens*, and the actinomycete *Nocardioopsis dassonvillei*.

TTX mainly concentrates in fish of the Tetraodontidae family, which includes puffer fish. TTX has also been found in xanthid crabs, horseshoe crabs, some frogs and newts, and the blue-ringed octopus. Puffer fish poisoning mainly occurs in Asia, where *fugu* is a delicacy. In the first half of the 20th century, in Japan approximately 100 deaths per year from ingestion of *fugu* were reported. The mortality rate substantially decreased with improved legislation of *fugu* preparation and marketing, although TTX poisoning remains the major cause of fatal food poisoning in Japan (Isbister and Kiernan, 2005).

### Pharmacokinetics/toxicokinetics

Although the pharmacokinetics in humans is not fully understood, serum concentrations of TTX fall rapidly and may be undetectable after 12–24 h. In contrast, TTX can be detected in urine for up to 5 days after ingestion (Isbister and Kiernan, 2005).

After single subcutaneous injection in rats, TTX was detected in kidneys, heart, liver, lungs, intestine, brain, and blood, reaching a peak in 20 min. The concentrations were highest in kidneys and heart, and they were lowest in brain and blood. A rapid appearance of high TTX concentration in the kidneys and a slow disappearance from these organs were observed, suggesting that an appreciable amount of unchanged toxin is excreted in the urine (Kao, 1966).

### Mechanism of action

TTX affects the nervous system by preventing the propagation of the nerve impulse. It blocks Na<sup>+</sup> conductance by extracellular binding at receptor-site 1 of Na<sup>+</sup> channels to occlude the outer pore and thereby prevent

access of monovalent cations, blocking the membrane depolarization (Isbister and Kiernan, 2005).

## Toxicity

### Human toxicity

Onset of TTX poisoning generally occurs within minutes, seldom more than 6 h after consumption of contaminated fish. TTX intoxication has been categorized into four stages of progression. Stage 1 includes oral paraesthesias with or without gastrointestinal symptoms. Stage 2 includes paraesthesias in other areas and motor paralysis. Stage 3 includes muscular incoordination, aphonia, dysphagia, respiratory distress, cyanosis, and hypotension. Stage 4 includes respiratory paralysis and severe hypotension. The mortality rate in TTX poisoning depends, among other things, on access to intensive care facilities. Patients who have not died within 24 h generally recover completely. As with PSP, symptoms of TTX poisoning generally are completely resolved within 1 or 2 days (Gessner, 2000).

### Experimental toxicity

#### Single administration

Although the mechanism of action of TTX is well known, only outdated and limited data on its toxicity in animals are available. The effects of TTX have been tested on a variety of animal species. After i.p. injection in mice, the minimal lethal dose of TTX was 8 µg/kg, whereas the LD<sub>50</sub> was estimated to be 10 µg/kg and doses ranging from 12 to 14 µg/kg killed all the mice. After oral and subcutaneous administration in mice, the LD<sub>50</sub> was 332 and 16 µg/kg, respectively (Kao, 1966). Similar results were observed in a study in which LD<sub>50</sub> values after i.p., subcutaneous, and oral administration in mice were determined to be 10.7, 12.5, and 532 µg/kg, respectively. In rabbits, the minimal lethal doses and the lethal doses for all the animals were respectively 5.3 and 3.1 µg/kg after intramuscular injection and 5.8 and 3.8 µg/kg after i.v. injection (Xu *et al.*, 2003). In almost all animal species, the observed symptoms were similar and comparable. The toxic effects involve mainly the peripheral neuromuscular system, with varying extent of paralysis. Furthermore, TTX is a highly potent emetic agent so that vomiting was frequently observed in both cats and dogs. Moreover, a pronounced and long-lasting hypotension and hypothermia were observed (Kao, 1966).

### Treatment

There is no antidote available for clinical use. To appropriately treat respiratory failure or cardiac effects, a careful

observation of the poisoned patient is recommended. Admission to an intensive care unit is necessary in moderate to severe cases to prevent complications. In case of severe poisoning, atropine can be used to treat bradycardia. Respiratory support may be necessary for 24–72 h. Because the patient may be fully conscious during the poisoning, sedation is important when the patient is paralyzed.

## Concluding remarks

Due to the severity of TTX intoxication, prevention is very important: public education is essential to reduce puffer fish poisoning (How *et al.*, 2003). In Japan, low mortality rates were achieved after the introduction of legislation related to the preparation and marketing of *fugu* (Isbister and Kiernan, 2005). The official method to detect TTX in puffer fish in Japan is the MBA (Yasumoto, 1991), as translated to English by Dr. Yotsu-Yamashita (Hungerford, 2006). In the United States, no regulatory limits for TTX have been established, but personal importation of puffer fish is prohibited (Gessner, 2000). Various other methods have been developed for detecting tetrodotoxin, including LC-MS/MS, as briefly reviewed by Taylor *et al.* (2008) in their description of a surface-plasmon resonance sensor for TTX.

## CIGUATOXINS AND MAITOTOXINS

In tropical regions, many species of fish may become toxic, provoking neurological, gastrointestinal, and, sometimes, cardiovascular symptoms when ingested. Eating these contaminated fish, humans can develop ciguatera, a marine fish poisoning that causes diverse and often long-lasting health problems. Although this poisoning is rarely fatal (0.1%), it has been estimated that more than 25,000 people are affected every year.

## Background

Ciguatera is characterized by moderate to severe gastrointestinal symptoms (vomiting, diarrhea, and abdominal cramps), neurological signs (myalgia, paraesthesia, cold allodynia, and ataxia), pruritus, and, less common, cardiovascular effects. Both gastrointestinal and neurological symptoms are typical of ciguatera, but the proportion of each type depends on the region. Gastrointestinal symptoms characterize the first stage of this poisoning and are predominant in Caribbean cases of ciguatera. Generally, they appear within 2–12 h of contaminated fish ingestion and are represented mainly by

nausea, vomiting, and diarrhea, but also by abdominal pain. Neurological symptoms predominate in the Pacific area and develop over 24 h, although onset can be very different, even in patients who eat the same fish. Cold allodynia, a dysesthesia that induces a burning sensation on contact with cold objects, is one of the most typical symptoms of ciguatera and is commonly incorrectly referred as “temperature reversal” (Isbister and Kiernan, 2005).

Persistent effects of ciguatera have been reported, including a case-control study. Reported clinical effects of chronic ciguatera (CC), which impacts approximately 5% of ciguatera victims, include fatigue, arthralgia, myalgia, headache, and pruritus. Immune response abnormalities seen in CC parallel the chronic systemic inflammatory response syndrome seen in several chronic diseases (Shoemaker *et al.*, 2010). Also, depression and anxiety have been associated with CC (Isbister and Kiernan, 2005). Cardiovascular symptoms, not very frequent, include bradycardia and hypotension.

Initially, this poisoning can present similar symptoms to those of diarrheic shellfish poisoning or microbial food poisoning, so anamnesis data are important. Mild ciguatera cases are often misdiagnosed as a common illness such as the flu. Whereas symptoms of cold allodynia are indicative of ciguatera, differential diagnosis of neuropathy can be needed (Glaziou and Legrand, 1994; Isbister and Kiernan, 2005).

Ciguatoxins (CTXs) enter the food chain through the epiphytic benthic dinoflagellate *Gambierdiscus toxicus*. This species is common in coral reef waters and lives at temperature ranging from 20 to 34°C, in conditions of low salinity and depths (3–15 m). Its growth is increased in places where there is reef degradation by human or natural factors. Different strains of *G. toxicus* produce chemically distinct lipo- and water-soluble toxins, named CTXs and maitotoxins (MTXs), respectively (Lehane and Lewis, 2000; Lewis, 2001). Because the actual role of MTX in ciguatera is unclear, the two groups of toxins are treated separately.

The accumulation of these toxins through the food chain involves the ingestion of *G. toxicus* by herbivorous fish that are eaten by carnivorous species. CTXs are concentrated both into viscera (e.g., liver, intestines, and gonads) and into fish muscle, whereas MTXs seem to be confined only to the viscera. Pacific and Caribbean reef-fish species associated with ciguatera include *Lutjanids* (red bass and snappers), *Serranids* (coral trout from the Great Barrier Reef, sea bass, and groupers), *Epinephelids* (cod, including flowery cod and spotted cod), *Lethrinids* (emperors and scavengers), *Muraenids* (moray eels), *Scombrids* (mackerel, including Spanish mackerel, and tunas), *Carangids* (jacks and scads), and *Sphyraenids* (barracuda) (Lehane and Lewis, 2000; Yasumoto, 2001; Isbister and Kiernan, 2005).

Ciguatera is endemic in subtropical and tropical regions of the western Indian and Pacific Ocean regions and in the Caribbean Sea (Lehane and Lewis, 2000). In 2004 and 2008–2009, ciguatera outbreaks occurred for the first time near the west coast of Africa, in the Canary Islands (Boada *et al.*, 2010), tentatively due to climate changes. Due to the increase in trade of fish, ciguatera is not confined to the tropics. Epidemiological characterization of ciguatera has been limited by the lack of laboratory tests to confirm the presence of the toxins (Lewis *et al.*, 2000). A role for marine cyanobacteria in ciguatera has also been suggested (Golubic *et al.*, 2010).

## CIGUATOXINS

Toxins responsible for ciguatera are the CTXs (Figure 69.3), which are lipid-soluble and heat-stable polyethers with a backbone composed of 10–14 rings transfused by ether linkages into a ladder-like structure reminiscent of the brevetoxins (PbTx). Structural differences have been found in CTXs isolated in different regions so that Pacific (P-CTXs) and Caribbean (C-CTXs) CTXs are usually referred to separately (Lehane and Lewis, 2000; Yasumoto, 2001). The Pacific CTXs are the most potent and the most studied CTXs, and thus they are referred to here omitting the Pacific Ocean (P-) designation. Pacific CTXs form CTX1B, which possesses the highest toxicity of all CTXs, and the total toxicity of a sample is often expressed in CTX1B equivalents. Even among the Pacific group of CTXs, there is considerable regional diversity among Pacific CTX congener profiles, as described by Yogi *et al.* (2011). Studies have clarified just how complex is the evolution of ciguatoxicity. For many years, it was believed that oxidation of the less toxic forms produced by the algae (CTX4A and CTX3C; Figure 69.4) by cytochrome enzymes in the liver of fish (Lehane and Lewis, 2000; Yasumoto, 2001; Cembella, 2003) was necessary to produce the more toxic, oxidized CTX forms implicated in Pacific region outbreaks of ciguatera (primarily CTX1B and also 51-hydroxyCTX3C). However, Yogi *et al.* reported that some of the oxidized forms of the CTXs also occur directly in *G. toxicus*, including 52-epi-54-deoxyCTX1B, 54-deoxyCTX1B, and 51-hydroxyCTX3C (Figure 69.4). Thus, the role of fish metabolism in the transformation and ultimate toxicity of CTXs in the food web must be reexamined.

### Pharmacokinetics/toxicokinetics

Only limited data are available on absorption, distribution, metabolism, and excretion of CTXs. Most of the

toxicokinetic information is not obtained from actual pharmacokinetic studies but often derives either from direct clinical observations or from schemes hypothesized on the basis of the chemical properties of CTXs. The oral absorption of these toxins should be complete or almost complete due to their lipophilicity; it has been hypothesized that CTXs can also penetrate the skin and mucous membranes due to the observed human effects after local exposure (Lehane and Lewis, 2000). CTXs are able to cross the placenta (Pearn *et al.*, 1982) and they are also excreted in breast milk, although hyperaesthesia of the nipples of a lactating mother may interfere with breast-feeding (Bagnis and Legrand, 1987). Blood collected from mice exposed to a sublethal dose of C-CTX-1 was found to contain toxin concentrations of 0.25 and 0.12 ng/mL 30 min and 12 h after exposure, respectively (Bottein Dechraoui *et al.*, 2005).

### Mechanism of action

CTXs increase cell Na<sup>+</sup> permeability by binding to site 5 neuronal voltage-sensitive sodium channels (Cestèle and Catterall, 2000), which open at normal resting membrane potential. Therefore, CTXs affect various Na<sup>+</sup>-dependent mechanisms to enhance membrane excitability. They also induce mobilization of intracellular Ca<sup>2+</sup> and provoke cell swelling. The effects of CTXs are most prominent in nerves (Lewis *et al.*, 2000). The action of ciguatoxins and their precursors is highly dependent on toxin structure, with gambiertoxin (P-CTX4B) having a greater impact on K<sup>+</sup> channels than on Na<sup>+</sup> channels (Schlumberger *et al.*, 2010).

### Toxicity

#### Human toxicity

Ciguatera outbreaks sometimes produce fatalities, even though only a few fatal cases of ciguatera are reported. Postmortem analysis shows acute visceral congestion with eosinophilic necrotic lesions in the liver at light microscope and ultrastructural changes in the sural nerve with swelling Schwann cells, axonal compression, and vesicular degeneration of myelin (Lehane and Lewis, 2000; Terao, 2000). CTX-1B poses a health risk at concentrations higher than 0.1 pg/g (Pearn, 2001), which is in relatively close agreement with an outbreak reported by Oshiro *et al.* (2010) at just 0.175 pg/g. In its typical form, ciguatera is characterized initially by the onset of intense vomiting, diarrhea, and abdominal pain within hours after fish ingestion, but the neurological symptoms tend to be the most distinctive and permanent ones. They include sensory disturbances, such as

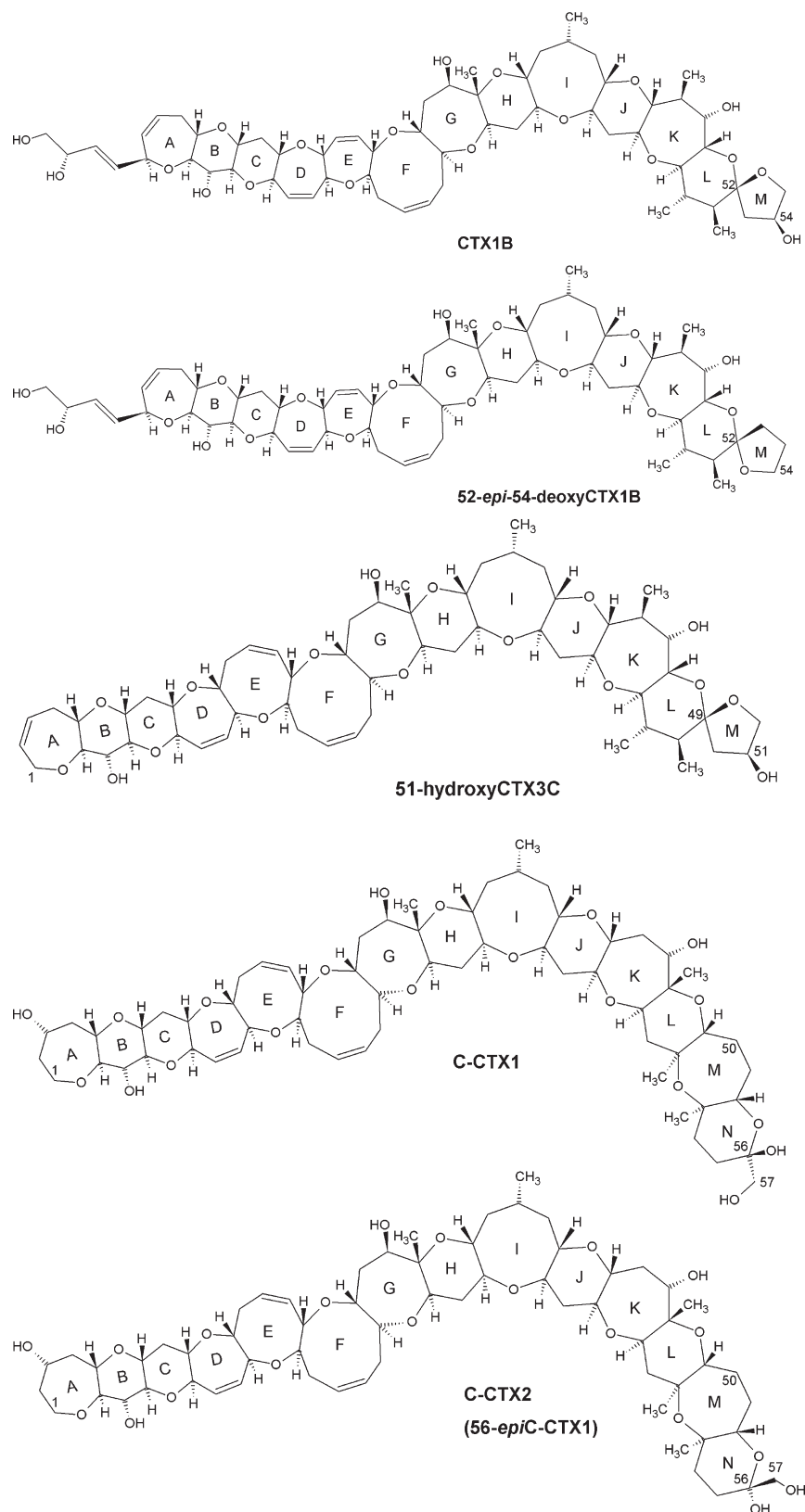
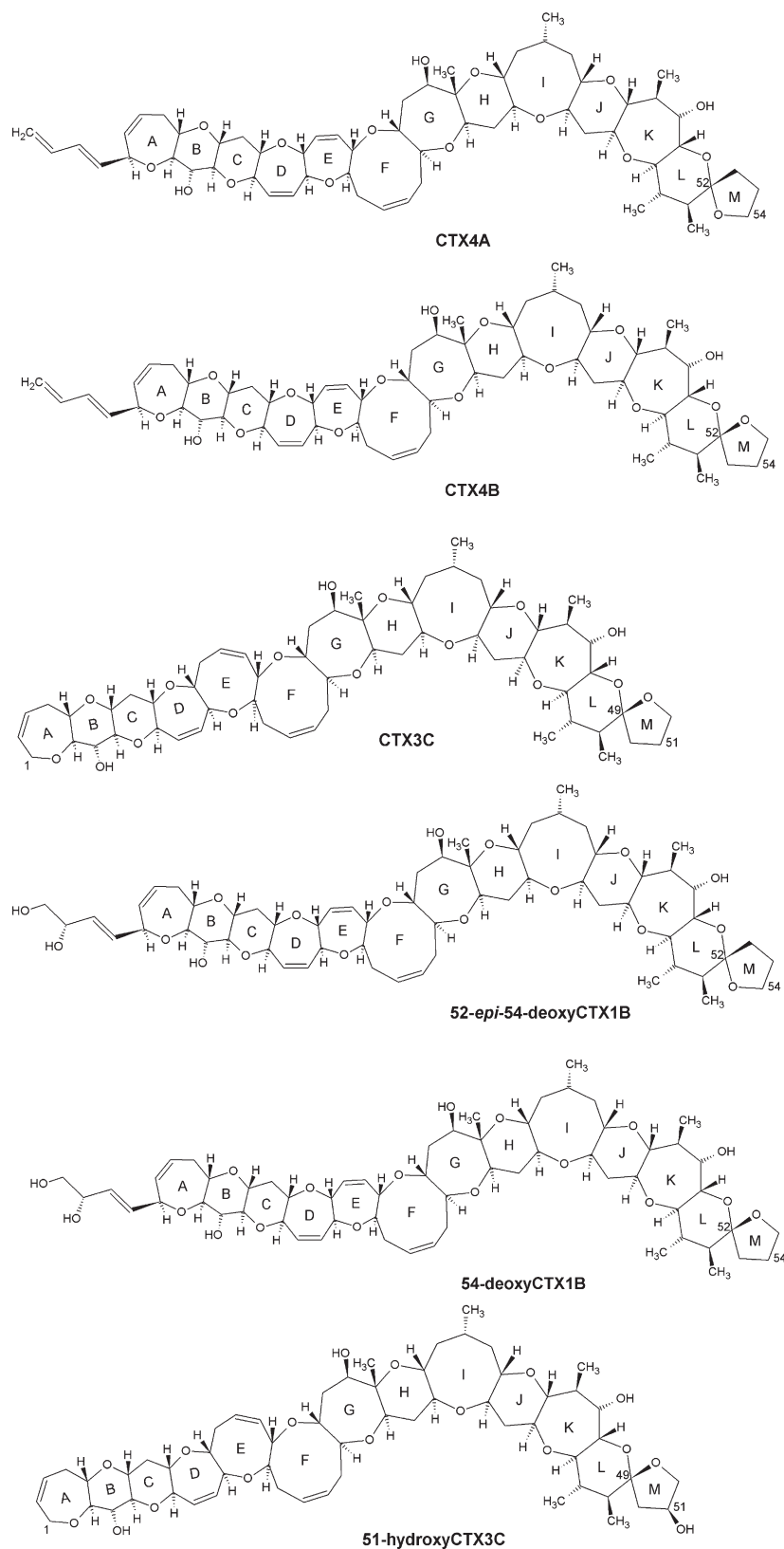


FIGURE 69.3 Chemical structure of ciguatoxins implicated in ciguatera outbreaks (from Dickey and Plakas, 2010, and Yogi *et al.*, 2011).





**FIGURE 69.4** Chemical structures of ciguatoxin congeners of note found in *Gambierdiscus toxicus* (from Dickey and Plakas, 2010, and Yogi *et al.*, 2011).

generalized pruritus, circumoral numbness, long-lasting weakness, and fatigue, and the unusual and perhaps pathognomic sensory discomfort triggered by cold stimuli ("cold allodynia"). Patients with bradycardia and/or hypotension may require urgent care because the infrequent cardiovascular symptoms may indicate a poor prognosis. It has been hypothesized that the cardiovascular effects can result from a positive inotropic effect and myocardium response to intracellular  $\text{Ca}^{2+}$  increase (Lehane and Lewis, 2000; Lewis *et al.*, 2000).

### Experimental toxicity

#### Single administration

After single administration in mice, toxicity is similar after either oral or i.p. administration, suggesting that oral absorption is almost complete in this species. P-CTXs and C-CTXs show a different potency by i.p. injection. The i.p.  $\text{LD}_{50}$  values in mice are 0.25, 2.3, and  $0.9\mu\text{g/kg}$  for P-CTX-1, -2, and -3, respectively, whereas  $\text{LD}_{50}$  values of 3.6 and  $1.0\mu\text{g/kg}$  were recorded for C-CTX-1 and -2. The acute i.p. injection or oral administration of P-CTX-1 or P-CTX-4C ( $0.7\mu\text{g/kg}$ ) in mice provoke similar toxicity, initially characterized by severe diarrhea. Target organs were heart, medulla of the adrenal glands, autonomic nerves, and penis. Light microscopy analysis shows marked swelling and focal necrosis of cardiac muscle cells. Degeneration of cells in the medulla of the adrenal glands was also observed. A marked lung edema, with congestion at alveolar spaces and bronchioles, was reported in mice with severe dyspnea. Continuous erection of the penis was observed in approximately 15% of the mice suffering from ciguatoxicosis (Lehane and Lewis, 2000; Terao, 2000). Hypothermia is also observed in mice, and toxicogenomics studies show that this is followed by activation of phase I and II detoxification pathways (Morey *et al.*, 2008). Using a murine macrophage model (Matsui *et al.*, 2010), CTXs have been shown to modulate mRNA expression of pro- and anti-inflammatory cytokines and of inducible nitric oxide synthase, and in bovine chromaffin cells they promote catecholamine secretion (Nguyen-Huu *et al.*, 2010).

#### Repeated administration

After repeated i.p. and oral administrations ( $100\text{ng/kg}$  of P-CTX-1 or P-CTX-4C) for 15 days, marked swelling of cardiac cells and endothelial lining cells of blood capillaries in the heart was observed. Although single doses of the same toxins did not provoke any discernible change at macroscopic, light microscopic, and even ultrastructural levels in the hearts of mice, the repeated administration of CTXs resulted in severe morphological but reversible cardiac changes. At the ultrastructural

level, the changes induced by repeated CTX administration were similar to those of mice receiving a CTX single dose ( $700\text{ng/kg}$  or more) (Terao, 2000). Bottein Dechraoui *et al.* (2008) related blood CTX levels to repeated administration of ciguatoxin to enhanced and sustained thermoregulatory, motor activity, and pain threshold and responses in mice.

### Treatment

For ciguatera poisoning, there is only supportive and symptomatic treatment, such as the control of fluid and electrolyte balance, because no effective antidote is available. The infrequent cardiovascular complications, such as symptomatic bradycardia and severe hypotension, may require treatment. The most common treatment during the acute phase is i.v. infusion of mannitol, but controversial data are reported about its efficacy: a double-blind, randomized, controlled trial found no difference between mannitol and saline treatment. Local anesthetics and antidepressants may also be useful (Lehane and Lewis, 2000; Lewis, 2001; Isbister and Kiernan, 2005).

### Concluding remarks

Because ciguatera remains a significant problem, prevention is important in endemic regions. The most widely used method for monitoring purposes is the MBA, based on clinical signs (marked hypothermia) and death observed for up to 48 h after i.p. injection of a 20-mg ether extract from fish muscle. Due to its poor sensitivity, this assay cannot detect the presence of CTXs in low-level contaminated ciguateric fish (Lehane and Lewis, 2000). Development of modern methods for detecting CTXs has been exceptionally challenging because the purified toxins are seldom available, and even then in minute quantities and generally not commercially. A rapid extraction procedure has been described for the LC-MS/MS detection of CTXs (Lewis *et al.*, 2009), and impressive gains have been made by Yogi *et al.* (2011) in the detection of 16 different CTXs forms using 12 toxin standards synthesized or isolated from nature. In the United States, ciguatera outbreaks are investigated (Dickey and Plakas, 2010) using a laboratory-based cytotoxicity screening, employing N2A neuroblastoma cells to screen for CTXs via (site 5) modulation of the VGSC (Manger *et al.*, 1993). Following N2A screening using a modified format, confirmation of CTXs contamination in positive samples is by LC-MS (Dickey and Plakas, 2010). Rapid assays have also been developed using neuroblastoma cells treated with voltage-sensitive dyes (Louzao *et al.*, 2004), and flow cytometry offers selection of the most responsive cells in

such assays, allowing for rapid estimation of CTX (site 5) toxicity in fish extracts (Manger *et al.*, 2007). Using flow cytometry, it is possible to directly observe depolarization of the N2A neuroblastoma cells by CTX (Manger *et al.*, 2007), contradicting assertions (LePage *et al.*, 2005) that neuroblastoma (N2A cells)-based cytotoxicity assays are insensitive to CTX without veratridine also present. Addition of veratridine enhances the signal obtained using CTX alone by three orders of magnitude, enabling high-sensitivity detection of the CTXs.

Although cytotoxicity and binding assays have been helpful in ciguatera research and various assays, including radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and binding and cytotoxicity assays, have been developed and reviewed (Dickey and Plakas, 2010), there is still a need for easy diagnostic and screening methods for CTXs. Attempts at developing a commercially available rapid test kit for CTXs have been plagued by false positives and negatives (Wong *et al.*, 2005) and have failed to be officially AOAC approved (Hungerford, 2009). Total synthesis of CTX3C and fragments of the predominant toxin CTX1B shows promise for supporting future development of a sandwich ELISA based on two synthetic fragments (Tsumuraya *et al.*, 2010). However, the cross-reactivity of the (CTX3C-derived) antibodies for CTX1B is not sufficient to allow the assay's application in screening toxic fish. In April 2011, the U.S. Food and Drug Administration established the first regulatory (advisory) levels for ciguatoxins at 0.01ng CTX1b equivalents/g fish for the Pacific and 0.1ng C-CTX1 equivalents/g fish for the Caribbean (Dickey and Plakas, 2010; FDA, 2011). These advisory levels were determined from assessment of human ciguatera outbreaks using data from variations of the N2A cytotoxicity assay (Manger *et al.*, 1993) combined with LC-MS/MS (Dickey and Plakas, 2010) and discussions between experts in the United States, Japan, and Australia.

## MAITOTOXINS

MTX, a water-soluble compound isolated together with CTXs, was considered as a biotoxin involved in ciguatera (Yasumoto, 2001). However, because its content is insignificant in fish flesh and it has low oral toxicity, MTX seems to play a limited role in ciguatera (Estacion, 2000; Yasumoto, 2000). MTX, also called MTX-1 (Figure 69.5), is a polycyclic polyether and, with the exception of biopolymers, the largest natural compound isolated and structurally elucidated to date ( $C_{164}H_{256}O_{68}S_2Na_2$ ; molecular weight (MW) = 3422) (Yasumoto, 2001). It is produced by the epiphytic dinoflagellate *Gambierdiscus toxicus* and accumulates in the liver of fishes such as surgeonfish and parrotfish. MTX

was first detected in viscera of *Ctenochaetus striatus* (maito), from which MTX derived its name. MTX analogs (MTX-2, MW = 3298; and MTX-3, MW = 1060) have been purified from *G. toxicus* strains (Holmes and Lewis, 1994; Bouaïcha *et al.*, 1997; Terao, 2000).

## Pharmacokinetics/toxicokinetics

No data on the absorption, distribution, biotransformation, and elimination of MTXs are available.

## Mechanism of action

MTX is a powerful activator of  $Ca^{2+}$  entry via nonselective cation channels in a wide variety of cells. Thus, it causes an increase in cytosolic  $Ca^{2+}$  levels that stimulates a broad spectrum of calcium-dependent processes, including cell death (Escobar *et al.*, 1998; Estacion, 2000; Morales-Tlalpan and Vaca, 2002).

## Toxicity

### Human toxicity

MTX toxicity in humans is unclear because its role in ciguatera is not completely understood (Estacion, 2000).

### Experimental toxicity

#### Single administration

After i.p. injection in mice, the  $LD_{50}$  of MTX was 500ng/kg (Yasumoto, 2000). An i.p. injection of MTX in mice (200–400ng/kg) and rats (400ng/kg) induced severe alterations at the gastric mucosa, cardiac muscle, and lymphoid tissues, as well as an increase in plasma cortisol, which was probably involved in the involution of lymphoid tissues (Terao, 2000). A hypothermic effect after i.p. injection in rats (338ng/kg) has also been reported (Gordon and Ramsdell, 2005).

#### Repeated administration

Repeated daily i.p. injection of MTX in mice (45ng/kg for 13 days) resulted in marked atrophy of lymphoid tissues, reduced circulating lymphocytes and serum immunoglobulin M levels, as well as increased calcium content of adrenal glands and plasma cortisol (Terao, 2000).

## Concluding remarks

Although MTX is accumulated mainly in viscera of herbivorous fish and seems to play a marginal role in

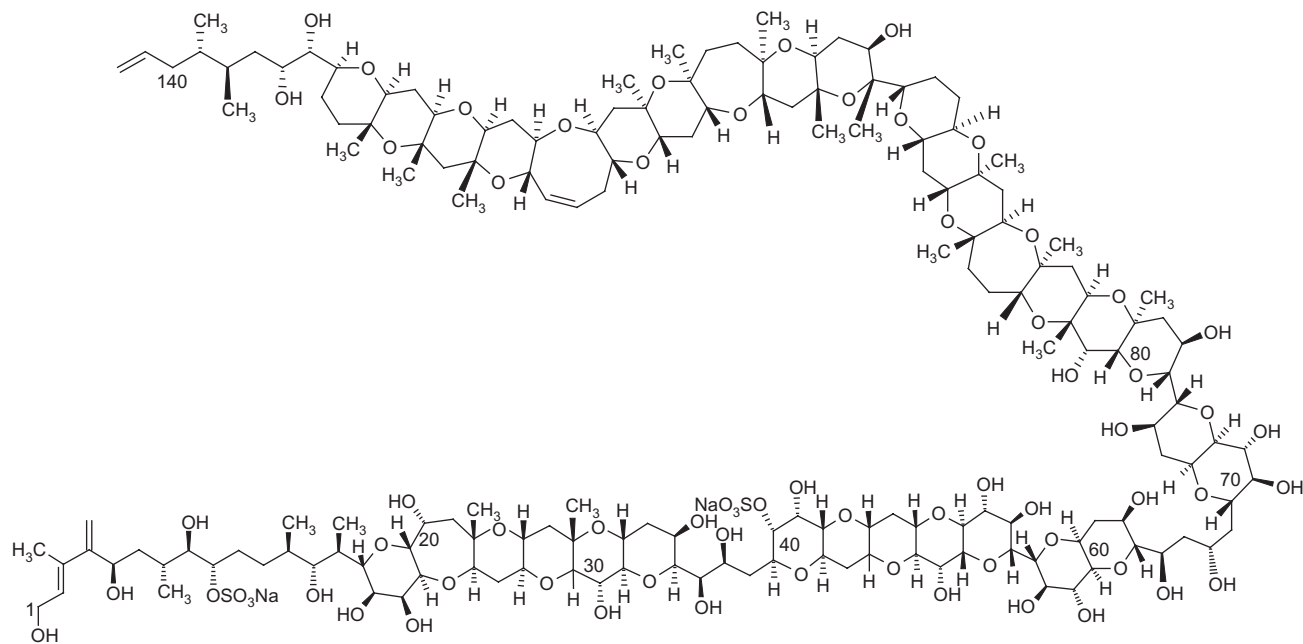


FIGURE 69.5 Chemical structure of maitotoxin (from Yasumoto, 2001).

ciguatera, its possible contribution should not be disregarded, considering the practice in some areas of eating small fish without eliminating their viscera (Yasumoto, 2000). Therefore, detection of MTXs in fish and phytoplankton is of particular importance, so biological and chemical methods based on HPLC and zone electrophoresis with ultraviolet and/or MS detection have been developed (Fessard *et al.*, 1994; Lewis *et al.*, 1994; Van Dolah *et al.*, 1994; Bouaïcha *et al.*, 1997). Although not demonstrated for seafood extracts or proposed analytically, it appears feasible that an assay could be designed based on the depolarization of synaptosomal plasma membrane by MTX and a voltage-sensitive dye as per the study of Tagialatela *et al.* (1990).

## DOMOIC ACID AND ANALOGS

### Background

Amnesic shellfish poisoning (ASP) is a toxic syndrome provoked by domoic acid (DA)-contaminated shellfish consumption. ASP differs from most other neurotoxic marine poisonings because the main effect is on the central nervous system (Isbister and Kiernan, 2005). It was reported for the first time in Canada in 1987 when, after consumption of contaminated mussels, people developed gastrointestinal symptoms within 24 h (vomiting, abdominal cramps, and diarrhea) and/or

neurological symptoms within 48 h (headache, loss of short-term memory, disorientation, lethargy, seizures, and, sometimes, convulsions and coma). Mortality was also recorded. Because the most relevant clinical effect was memory loss, the condition was termed ASP. The loss of memory in patients intoxicated with DA-contaminated mussels appeared to be similar to that of patients with Alzheimer's disease. However, whereas symptoms of Alzheimer's disease are generally present in older people and intensify with advancing age, loss of memory in mussel-intoxicated patients is not affected by patient age. Furthermore, the finding that higher cortical functions, such as intellect, were not affected by DA poisoning distinguished ASP from Alzheimer's disease (Nijjar and Nijjar, 2000; Jeffrey *et al.*, 2004).

The toxin responsible for ASP is DA (Figure 69.6), an amino acid of the kainoid class of compounds and 10 DA isomers (isodomoic acids A–H and DA 5'-diastereoisomer) that are less toxic than the parent compound. DA and its isomers are water-soluble and heat-stable tricarboxylic amino acids. Initially, DA was only known from the red algae *Chondria armata*. After 1987, *Pseudonitzschia* marine diatoms, such as *P. multi-series*, *P. pseudodelicatissima*, and *P. australis*, were shown to produce DA. *Pseudonitzschia navis-varengica* has been found to produce isodomoic acids A and B as main toxins (Kotaki *et al.*, 2005).

DA has been shown to accumulate in blue mussels (*Mytilus edulis*, *M. galloprovincialis*, and *Perna canaliculus*) as well as in other shellfish, such as cockles (*Cerastoderma*



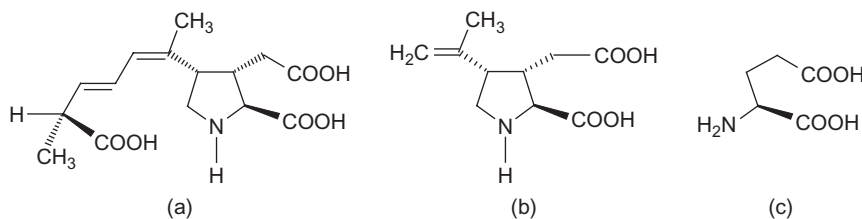


FIGURE 69.6 Chemical structure of (a) domoic acid, (b) kainic acid, and (c) glutamic acid (from Nijjar and Nijjar, 2000).

*edule*), razor clams (*Siliqua patula*), scallops (*Pecten maximus*), and crabs (*Cancer magister*), and also in furrow shell (*Scrobicularia plana*) and anchovies (Jeffery *et al.*, 2004). DA-contaminated shellfish and potentially toxic *Pseudonitzschia* ssp. have been detected worldwide. Although DA has caused bird (via toxic anchovies) and other animal mortalities, the only toxic episode with confirmed human illnesses is that which occurred in Canada.

### Pharmacokinetics/toxicokinetics

Very limited data are available on the absorption, distribution, metabolism, and excretion of DA, showing no appreciable differences in the effects observed after i.p. and oral administration. It has been suggested that DA is poorly absorbed after the usual exposure route (Jeffery *et al.*, 2004). Furthermore, in the two studies comparing i.p. and oral administration in rodents, involving a small group size ( $N = 51$  or 2/dose) (Iverson *et al.*, 1989), DA was almost completely excreted in the feces, supporting the hypothesis that DA is not well absorbed. Truelove *et al.* (1996) reported that only 2% of the orally administered dose of DA (5 mg/kg/day) in rats ( $N = 510$ ) was excreted in the urine. Similar results (mean DA urinary excretion of the daily dose ranged from 4 to 7%) were obtained in cynomolgus monkeys ( $N = 53$ ) treated with a subtoxic dose of DA for 32 days (Truelove *et al.*, 1997). Once absorbed, DA appears to be poorly metabolized. In fact, approximately 75% of a dose of  $^3\text{H}$ -labeled DA i.v. injected in rats ( $N = 58$ ) was excreted unchanged in the urine within 160 min, suggesting that it is not significantly metabolized before rapid elimination. In the same study, metabolism to compounds of greater hydrophilicity was hypothesized to be due to remaining radiolabel eluted earlier than DA during LC separation (Suzuki and Hierlihy, 1993). In another study on the distribution and excretion of i.v. administered DA in rats (500 and 1000  $\mu\text{g/kg}$ ) and monkeys, half-lives of approximately 20 and 110 min, apparent volume of distribution of approximately 300 and 180 ml/kg, and apparent clearances of approximately 10 and 1 mL/min/kg were reported. Although a limited number of animals were included in the study, these data suggest that i.v. injected DA is well

distributed into the body fluids in both species and is rapidly eliminated (Truelove and Iverson, 1994).

After DA i.v. administration in rats, DA poorly permeates across the blood–brain barrier and appears not to cross via a specific transport carrier (Preston and Hynie, 1991). However, despite this limited transfer across the blood–brain barrier, the brain is the primary organ of DA-induced toxicity.

### Mechanism of action

The toxicity of DA in the nervous system is known to occur on excitatory amino acid receptors and on synaptic transmission. Two amino acids, L-glutamate and L-aspartate, are considered to be neurotransmitters and act upon several receptor types. Three receptor subtypes have been described for excitatory amino acids, with the kainic acid (KA) and the N-methyl-D-aspartate receptors being best characterized.

DA produces its action through pre- and postsynaptic receptors in a manner similar to KA opening the channel to  $\text{Ca}^{2+}$  and inducing cellular lethality. Due to the structural similarity of DA to glutamic acid and, in particular, to KA (Figure 69.6), DA has a strong affinity for subclasses of kainate receptors. Kainate receptors are widely distributed in the mammalian brain and are particularly concentrated in the CA1–CA3 regions of the hippocampus in rodents, in the CA2 and CA3 regions in nonhuman primates, and in the CA3 region in humans. The high affinity of DA to kainate receptors and the apparent co-localization of these receptors at sites where DA induced damage in the brains of rodents and primates suggest that DA–kainate receptor interactions play a major role in the toxic response. This binding of DA to glutamate receptor subtypes appears to stimulate neuronal firing, eliciting an excitatory response both *in vitro* and *in vivo*. Although the exact mechanism of neuronal stimulation leading to tissue brain damage is not completely understood, an influx of  $\text{Ca}^{2+}$  into cells was observed in brain tissue slices exposed to DA, and increased cytosolic  $\text{Ca}^{2+}$  levels in hippocampal pyramidal neurons exposed to DA were reported. It seems that DA binding to glutamate receptors in specific brain regions leads to excitation of neurons, giving rise to an

influx of  $\text{Ca}^{2+}$  resulting in a failure to maintain intracellular ion homeostasis and in cell death (Jeffery *et al.*, 2004).

## Toxicity

### Human toxicity

The ASP outbreak occurred in 1987 in Canada after the mussel (*M. edulis*) ingestion was characterized by gastrointestinal and unusual neurological symptoms. Although 150 reports of this illness were recorded, only 107 individuals met the clinical definition of the poisoning (Jeffery *et al.*, 2004). The most common gastrointestinal symptoms were vomiting (76%), abdominal cramps (50%), and diarrhea (42%); the most common neurological symptoms were headache (43%) and loss of short-term memory (25%) (Nijjar and Nijjar, 2000).

The poisoning was particularly severe in 19 people who were hospitalized: 12 individuals with particularly severe symptoms (e.g., seizures, coma, profuse respiratory secretions, or unstable blood pressure) required treatment in the intensive care unit. Although in most patients, decreased arousal and somnolence were seen, in some cases leading to coma, in less severely affected patients, agitation was observed. Cardiovascular symptoms (tachycardia, hypotension, and arrhythmias) may have been a consequence of dysfunction of the central autonomic centers because there was no evidence of primary cardiovascular impairment. In most patients, symptoms resolved in a few weeks, but residual memory impairment can persist (Doble, 2000).

Three hospitalized patients died 11–24 days after consumption of mussels, and a fourth patient died of myocardial infarction within 3 months. Postmortem histological examination showed neuronal necrosis and astrocytosis, most prominent in the hippocampus and amygdala nucleus (Nijjar and Nijjar, 2000).

In nine cases of poisoning, estimates of DA exposure were derived from analysis of leftover mussels collected from households or restaurants. The amount of DA ingested ranged from 60 to 290 mg in poisoned patients. Thus, it seems that 60 mg DA/person (<1 mg/kg) is sufficient to provoke gastrointestinal symptoms, whereas ingestion of 270 mg DA/kg (<4.5 mg/kg) provokes neurological effects (Jeffery *et al.*, 2004).

### Experimental toxicity

#### Single administration

After i.p. administration in mice, LD<sub>50</sub> values of 2.4 and 3.6 mg DA/kg were reported in two different studies using DA-contaminated mussel extracts. Clinical symptoms such as scratching, tremors, and seizures at both

lethal and sublethal doses of the extracts were observed. LD<sub>50</sub> values have been recorded for newborn mice after i.p. administration at different postnatal days: the results suggest that they may be much more sensitive to DA than are adult mice (Jeffery *et al.*, 2004).

Neuropathological studies were carried out in both mice and rats. Dose-related lesions in the brains (edema in the hypothalamus and hypothalamic arcuate nucleus and neuronal degeneration in different areas of the hippocampus) of mice and rats were found after i.p. and after *per os* administration of mussel extracts containing DA (Iverson *et al.*, 1989).

Similar effects have been reported in studies of nonhuman primates after i.p. and i.v. injection. Severe vomiting and scratching were reported after single i.v. DA administration (0.24, 0.5, 1.0, 1.25, 1.5, 2, and 4 mg/kg) in cynomolgus monkey, with a dose-related latency in the appearance of the symptoms. DA doses greater than 1.0 mg/kg provoked death by respiratory failure in four of seven animals. Postmortem examination revealed lesions in the hippocampus (Scallet, 1995), and in a similar study utilizing specialized histochemistry, the thalamus also appeared to be affected (Schmued *et al.*, 1995).

#### Repeated administration

After repeated (64 days) *per os* exposure to DA (0.01 or 5 mg/kg/day) of male and female rats, neither clinical abnormalities nor differences in hematology, clinical chemistry, or histopathology were observed in treated animals (Jeffery *et al.*, 2004). Neither clinical symptoms nor significant changes in body weight, hematology, clinical chemistry, or brain histology were observed in cynomolgus monkey orally treated with low doses of DA (0.5 mg/kg/day for 15 days and 0.75 mg/kg/day for a further 15 days) (Truelove *et al.*, 1997).

#### Teratogenicity

The effects of DA (0–2 mg/kg) injected daily i.p. to pregnant rats from gestational days 7 to 16 were studied both on mothers and offspring at gestational day 22. Mother death was recorded only at the higher DA doses (6/12 and 6/9 at 1.75 and 2.0 mg/kg, respectively). At doses greater than 0.5 mg/kg/day, a reduction in live fetus number at term was recorded, but the number of deaths was not increased dose dependently. A nonsignificant increase in the number of fetuses with visceral/skeletal anomalies was reported (Khera *et al.*, 1994).

#### Genotoxicity

Because the structure of DA contains a butadiene moiety, there is the possibility for the formation of DNA-reactive epoxides *in vivo*. Although only limited data are available about its possible genotoxicity, DA (87 or 174  $\mu\text{M}$ )

did not increase mutation frequency in V79 Chinese hamster lung cells *in vitro* measured by thioguanine or ouabain resistance, sister chromatid exchange, or micronucleus assays, nor does it give rise to DNA-reactive metabolites (Jeffery *et al.*, 2004). *In vivo* data on DA carcinogenicity are not available.

## Treatment

The diagnosis is based on the history of ingestion of bivalve mollusks followed by characteristic symptoms. Environmental surveillance programs of phytoplankton in risk regions may be suggestive of a possibility of poisoning. Due to the risk of convulsions, emesis should not be induced. Severe cases should be admitted to the intensive care unit and monitored for convulsions, central nervous system depression, cardiovascular collapse, or gastric hemorrhages. Treatment is symptomatic, and no antidote is available (Gulland *et al.*, 2002).

## Concluding remarks

After the human outbreak of ASP in 1987, a regulatory limit for shellfish flesh was introduced in Canada and consequently in other countries. The limit, 20 mg/kg, is based on a retrospective estimate of the DA amount in mussels that resulted in human illness in the 1987 Canadian ASP outbreak and incorporates a 10-fold safety factor. Due to the worldwide distribution of the diatoms producing DA, the action limit employed by Canada has been adopted elsewhere and is the limit enforced in the European Union, the United States, New Zealand, and Australia (Jeffery *et al.*, 2004). An HPLC-UV method has gained favor in various countries (FAO/IOC/WHO, 2005).

# BREVETOXINS

## Background

Algal brevetoxins (PbTx) are neurotoxic polyethers produced by the dinoflagellates of *Karenia* genus (mainly *K. brevis*, formerly known as *Gymnodinium breve* or *Ptychodiscus breve*), which forms "red tide" blooms along the Florida coast and the Gulf of Mexico. These harmful blooms cause mass mortality of fish and other marine organisms. They also cause episodes of respiratory distress in humans after inhalation of the sea spray and eye and skin irritation after swimming in the sea. Dolphin and manatee mortalities have been traced to PbTx in the marine food web (Flewelling *et al.*, 2005).

Because shellfish are resistant to PbTx and accumulate these compounds, their ingestion may provoke an illness called neurotoxic shellfish poisoning (NSP) (Landsberg, 2002). NSP is characterized by acute gastrointestinal and neurological symptoms, including nausea, vomiting, diarrhea, chills, sweats, headache, muscle weakness and joint pain, paraesthesia, arrhythmias, difficult breathing, mydriasis, double vision, and troubles in talking and swallowing. Recovery occurs in 2 or 3 days, and no fatal cases from NSP have been reported (Baden and Adams, 2000; Hallegraeff, 2003; Isbister and Kiernan, 2005).

The PbTx are lipid-soluble and heat-stable polycyclic ether compounds grouped into two types (A and B) according to their backbone structures consisting of 10 (type A) or 11 (type B) transfused rings, and additional forms exist in which the lactone (A ring) is open (Figure 69.7). At least 15 PbTx can be found in cultures and natural blooms of *K. brevis* (Figure 69.7) (Plakas and Dickey, 2010). Metabolism of these toxins within shellfish, first reported by Ishida *et al.* (2004), is now known to be extensive (Plakas and Dickey, 2010), producing a variety of brevetoxin metabolites, many of them more polar than the PbTx (Figure 69.8).

The brevetoxin metabolites found in molluscan shellfish have widely ranging acute mouse i.p. toxicities, as summarized by Plakas and Dickey (2010). Some of these conjugates are more toxic than the algal forms; for example, metabolites BTX-B1 and BTX-B4 are respectively four and two times more toxic than their precursor, PbTx-2. Oral toxicity data are not available for any of the brevetoxin shellfish metabolites because isolation of these toxins and their synthesis have been challenging (Plakas and Dickey, 2010). Following an NSP outbreak, additional brevetoxin metabolites have also been identified in human urine (Figure 69.9). Other *Karenia* species were involved in NSP, whereas some raphidophytes (*Chattonella marina*, *C. antiqua*, *Fibrocapsa japonica*, and *Heterostigma akashiwo*) were reported to produce brevetoxin-like compounds, but no documented cases of NSP were caused by these species (Landsberg, 2002; Hallegraeff, 2003; Ciminiello and Fattorusso, 2004). *Karenia* blooms have occurred mainly in the Gulf of Mexico, where NSP has historically been limited, but occasional blooms associated with NSP have also been reported along the mid- and south Atlantic coast of the United States and in New Zealand. Shellfish involved in NSP are mainly oysters, clams, cockles, and mussels (Landsberg, 2002).

## Pharmacokinetics/toxicokinetics

After oral administration of [<sup>3</sup>H]-labeled PbTx-3 to rats, the toxin was shown to distribute to all organs and concentrated mainly in the liver. It was eliminated in

A-type brevetoxin backbone	R	Lactone ring A opened, A-type backbone
<b>PbTx-1</b>	$\text{CH}_2\text{C}(\text{=CH}_2)\text{CHO}$	<b>Open A-ring PbTx-1</b>
<b>PbTx-7</b>	$\text{CH}_2\text{C}(\text{=CH}_2)\text{CH}_2\text{OH}$	<b>Open A-ring PbTx-7</b>
<b>PbTx-10</b>	$\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{OH}$	
<b>Oxidized PbTx-1</b>	$\text{CH}_2\text{C}(\text{=CH}_2)\text{COOH}$	<b>Open A-ring, oxidized PbTx-1</b>

B-type brevetoxin backbone	R	Lactone ring A opened, B-type backbone
<b>PbTx-2</b>	$\text{CH}_2\text{C}(\text{=CH}_2)\text{CHO}$	<b>Open A-ring PbTx-2</b>
<b>PbTx-3</b>	$\text{CH}_2\text{C}(\text{=CH}_2)\text{CH}_2\text{OH}$	<b>Open A-ring PbTx-3</b>
<b>PbTx-6</b>	<b>H-ring epoxide of PbTx-2</b>	
<b>PbTx-9</b>	$\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{OH}$	
<b>Oxidized PbTx-2</b>	$\text{CH}_2\text{C}(\text{=CH}_2)\text{COOH}$	<b>Open A-ring, oxidized PbTx-2</b>

FIGURE 69.7 Chemical structure of brevetoxins found in natural blooms and cultures of *Karenia brevis* (from Dickey and Plakas, 2010).

equivalent quantities through urine and feces: approximately 80% of the dose was excreted within 7 days (during the first 48h, PbTx-3 was cleared mainly through the feces, and afterward it was cleared mostly through urine) (Cattet and Geraci, 1993).

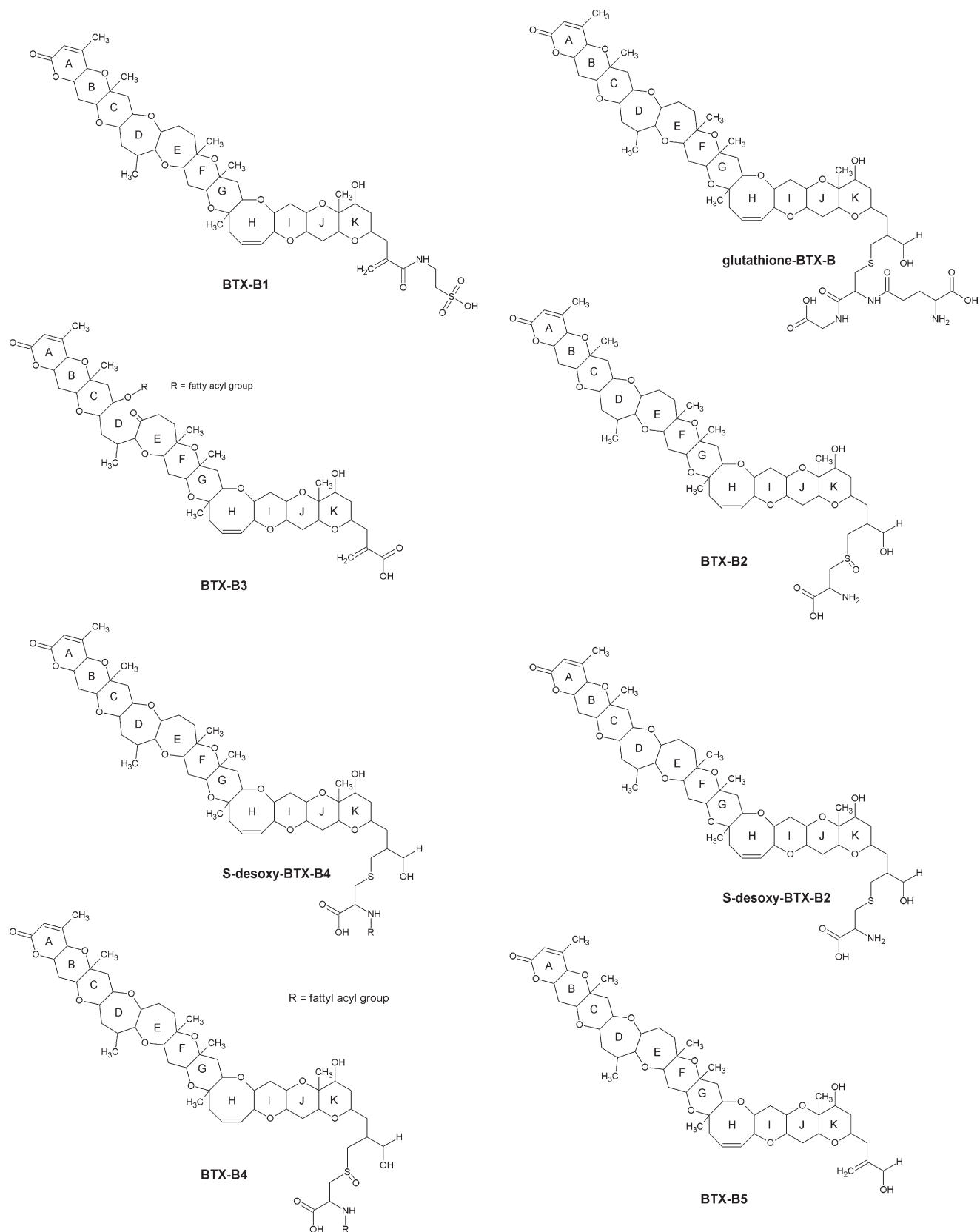
After i.p. injection of PbTx-3 in mice, blood concentration of the toxin was maximal between 0.5 and 4h. After 24 h, it was reduced by one-third, and it was still detectable after 7 days. A significant part of PbTx-3 in mouse plasma binds to high-density lipoproteins (Fahey *et al.*, 2001; Woofter *et al.*, 2003, 2005). In rats, after i.p. injection of PbTx-2 and PbTx-3, the toxins were detected in blood within 1h, and for PbTx-2, a rapid metabolism to polar cysteine conjugates, eliminated in urine over 24h, was observed (Radwan *et al.*, 2005).

An i.v. administration of  $^3\text{H}$ -labeled PbTx-3 to rats showed that the toxin cleared rapidly from the blood (<10% remained after 1min) and distributed mainly to the liver, skeletal muscle, and gastrointestinal tract (18, 70, and 8% of the dose after 30min, respectively). Within 24h, PbTx-3 concentration in skeletal muscle decreased to 20% of the dose, whereas that in liver

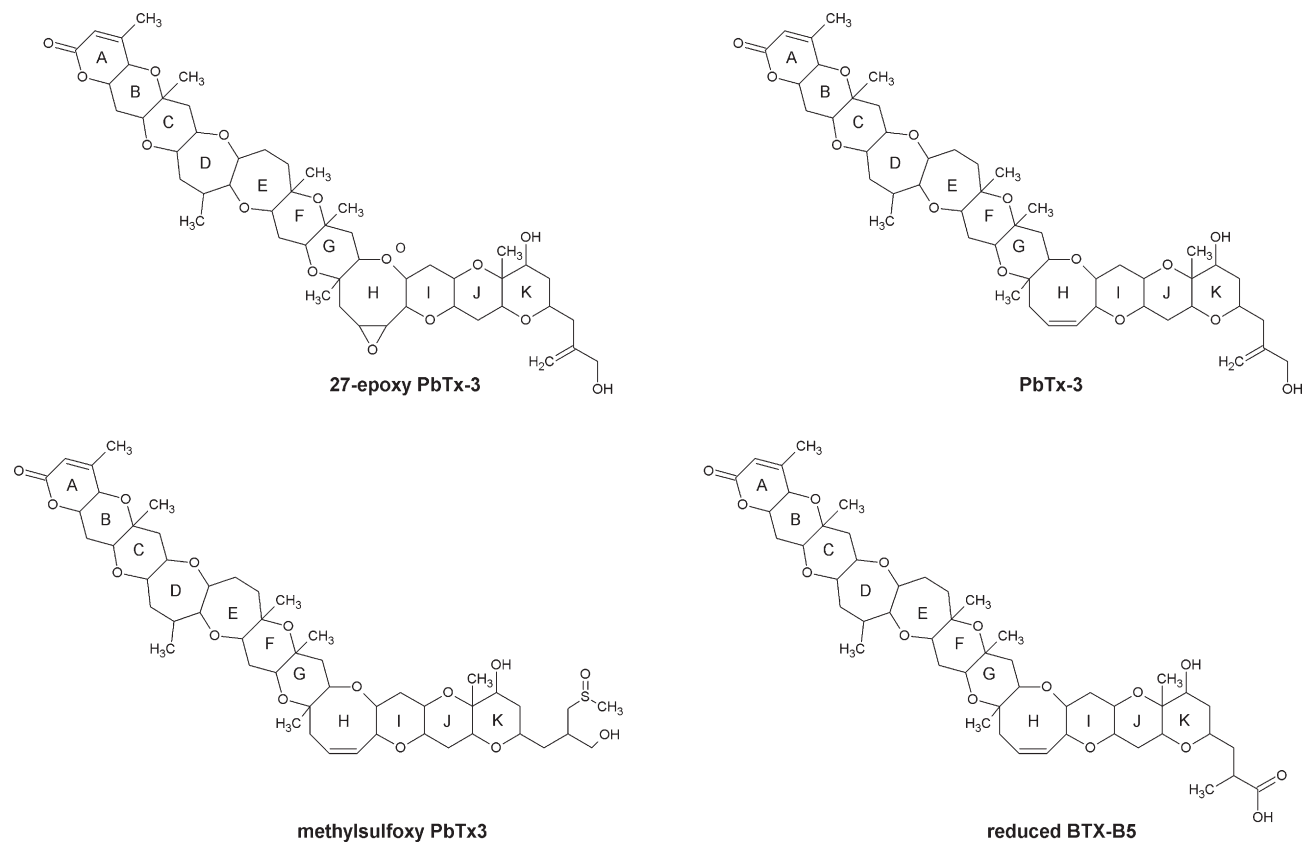
remained constant and increased in the gastrointestinal tract and feces, probably because of biliary excretion. By day 6, approximately 14% of the toxin had been excreted through urine and 75% in feces, also in the form of more polar metabolites (Poli *et al.*, 1990).

Intratracheal instillation of [ $^3\text{H}$ ]-labeled PbTx-3 to rats revealed that more than 80% of the dose was cleared within 0.5h from the lung and distributed throughout the body, mainly to the carcass (49%), intestine (32%), and liver (8%). Blood, brain, and fat contained the lowest levels of the toxin. Approximately 20% of the initial level in tissues was retained for 7 days. The majority of PbTx-3 was excreted within 48 h in feces (60%) and urine (30%) (Benson *et al.*, 1999). Also in mice, after intratracheal instillation, PbTx-3 distributed rapidly to tissues, mainly in liver and gastrointestinal tract, whereas approximately 90% of excretion occurred within 4 days in urine (11%) and feces (64%) (Tibbetts *et al.*, 2006). After repeated inhalation of PbTx-3 by rats for 5 or 22 days, small amounts of the toxins were detected in splenic and peribronchiolar lymphoid tissue as well as in liver, in which no accumulation of brevetoxin occurred (Benson *et al.*, 2004, 2005).





**FIGURE 69.8** Chemical structure of molluscan shellfish metabolites of the brevetoxins (PbTx-2 and PbTx-3 also occur and are shown in Figure 69.7; from Dickey and Plakas, 2010).



**FIGURE 69.9** Chemical structure of brevetoxins and metabolites found in human urine following an NSP outbreak (from Dickey and Plakas, 2010).

Cutaneous application of  $^3\text{H}$ -labeled PbTx-3 on pig skin revealed a rapid penetration of the toxin to the dermis, with maximal dermal accumulation at 4h (Kemppainen *et al.*, 1991).

## Mechanism of action

PbTxS and BTXs bind to site 5 on the  $\alpha$  subunit of voltage-sensitive sodium channels in the cell membranes (Cestèle and Catterall, 2000). Normally, these channels open in response to membrane depolarization and subsequently inactivate, returning to closed configuration during the membrane repolarization. Toxin binding opens the voltage-sensitive sodium channels due to a negative shift in activation potential resulting in a sustained influx of  $\text{Na}^+$  and membrane depolarization. There is also alteration of the normal changes of the voltage-sensitive sodium channels configuration during the depolarization/repolarization processes. These actions affect the membrane properties of excitable cells and are the basis of the neurotoxic effects of the toxins. A structural feature of these compounds (Figure 69.7) required for this activity is the lactone in the A ring (“head” of

the molecule) as well as the conserved structure on the “tail” rings. Receptor binding studies with synaptosome preparations from manatee brain showed tritiated PbTx3 binding with an affinity somewhat lower than that of rat and similar to that of fish excitable tissues (Trainer and Baden, 1999; Ciminiello and Fattorusso, 2004).

Respiratory problems associated with the inhalation of aerosolized PbTxS are believed to be due in part to opening of sodium channels. Other actions involved in the bronchoconstriction and/or the immunological effects at the respiratory tract seem to be related to stimulation of neurotransmitter release and mast cell degranulation, as well as to an inhibition of the phagocytic cell lysosomal proteinases known as cathepsins (Abraham *et al.*, 2005; Baden *et al.*, 2005).

## Toxicity

### Human toxicity

Ingestion of PbTxS and their metabolites in contaminated shellfish can cause NSP, a syndrome that includes some symptoms similar to ciguatera but less severe. The symptoms occur within 30min to 3h, last a few

days, and include nausea, vomiting, diarrhea, chills, sweats, headache, muscle weakness and joint pain, paraesthesia, arrhythmias, difficulty breathing, mydriasis, double vision, and trouble in talking and swallowing. Sometimes, coma occurs, but no mortality or chronic symptoms have been reported (Baden and Adams, 2000; Isbister and Kiernan, 2005).

Due to the fragility of *Karenia* cells, PbTxS can be released in seawater and aerosolized by wind and surf, with the possibility of inhalation exposure to these compounds, causing respiratory distress and irritation of the eyes and of respiratory tract mucosa. Normally, these symptoms are rapidly reversible by leaving the beach area. The main brevetoxin responsible for respiratory discomfort seems to be PbTx-3. During swimming, direct contact with toxic blooms and consequent skin, nasal, and eye irritation can occur (Landsberg, 2002).

### Experimental toxicity

#### Single administration

After i.p. injection in mice, the LD<sub>50</sub>s of PbTx-1, -2, and -3 were calculated to be 100, 200, and 170 µg/kg, respectively. The symptoms include immediate irritability, followed by hindquarter paralysis, dyspnea, salivation, lachrymation, urination, defecation, and death from respiratory paralysis (Landsberg, 2002; FAO/IOC/WHO, 2005).

The oral LD<sub>50</sub> in mice ranges from 520 µg/kg for PbTx-3 to 6600 µg/kg for PbTx-2 (FAO/IOC/WHO, 2005). PbTx-3 caused tremors followed by marked muscular contractions or fasciculations, tail elevation, labored breathing, and death (van Apeldorn *et al.*, 2001).

#### Repeated administration

Studies on repeated exposure to PbTxS were carried out in rats after inhalation of PbTx-3. Exposure to 500 µg PbTx-3/m<sup>3</sup> by nose-only inhalation for 0.5 or 2 h/day for 5 days (corresponding to 8.3 and 33 µg/kg/day, respectively) provoked a reduction of body weight at the highest dose, but no tissue lesions or signs of cytotoxicity and inflammation in bronchoalveolar lavage fluid were observed. In contrast, the humoral-mediated immunity was suppressed. A more prolonged inhalation exposure of rats (22 days) to PbTx-3 (corresponding to 0.9 and 5.8 µg/kg/day, respectively) showed similar results: reduced body weight in both PbTx-3 dosed groups of rats, suppression of humoral-mediated immunity, as well as minimal alveolar macrophage hyperplasia and increased blood reticulocytes (Benson *et al.*, 2004, 2005).

### Toxicity for fish and other marine animals

PbTxS are potent ichthyotoxins, being responsible for the deaths of billions of fish throughout the years. These toxins are thought to be absorbed through the gills, but

ingestion and mortality can also occur in the presence of approximately 250 *K. brevis* cells/mL. Signs of intoxication in fish include violent twisting and corkscrew swimming, defecation and regurgitation, pectoral fin paralysis, caudal fin curvature, loss of equilibrium, quiescence, vasodilatation, convulsions, and death due to respiratory failure. Chronically intoxicated fish show little pathology aside from slight precipitate hemolysis. Chronic hemolysis was detected via anemia, cyanosis, viscous blood, splenomegaly, hepatic hemosiderosis, and dehydration. Frequently, birds are also found moribund or dead, particularly double-crested cormorants, red-breasted mergansers, and lesser scaup, as are dolphins and manatees (Landsberg, 2002).

### Treatment

The treatment is symptomatic and supportive. Recovery occurs in 2 or 3 days (van Apeldorn *et al.*, 2001).

### Concluding remarks

In the United States and other countries, the regulatory level of 80 µg of PbTx-3 equivalents/100 g shellfish was established. Monitoring for NSP toxins is performed by *in vivo* MBA based on the observation of the survival time of mice after i.p. injection of an ether extract of shellfish. Brevetoxins are extensively metabolized in shellfish, and ELISA and LC-MS/MS alternatives for the monitoring of shellfish for the brevetoxins are being validated (Plakas and Dickey, 2010).

## PALYTOXIN AND ITS ANALOGS

### Background

Palytoxin (PLTX) is one of the most toxic nonprotein compounds known to date, originally isolated from the coral *Palythoa toxica* in Hawaii. It is a water-soluble compound characterized by a long, partially unsaturated aliphatic polyhydroxylated backbone with spaced cyclic ethers, 64 chiral centers, and two amide groups (Figure 69.10). PLTX and a series of its analogs, such as homoPLTX, bishomoPLTX, neoPLTX, deoxyPLTX, and 42-hydroxyPLTX, were subsequently identified in *Palythoa* species. Moreover, PLTX and its analogs, including ostreocin-d, mascarenotoxin-a, -b and -c, and ovatoxin-a, -b, -c, -d, and -e, were identified in benthic dinoflagellates of the genus *Ostreopsis*, which have been proposed as producing organisms even though a bacterial origin has been suggested (Katikou, 2008;

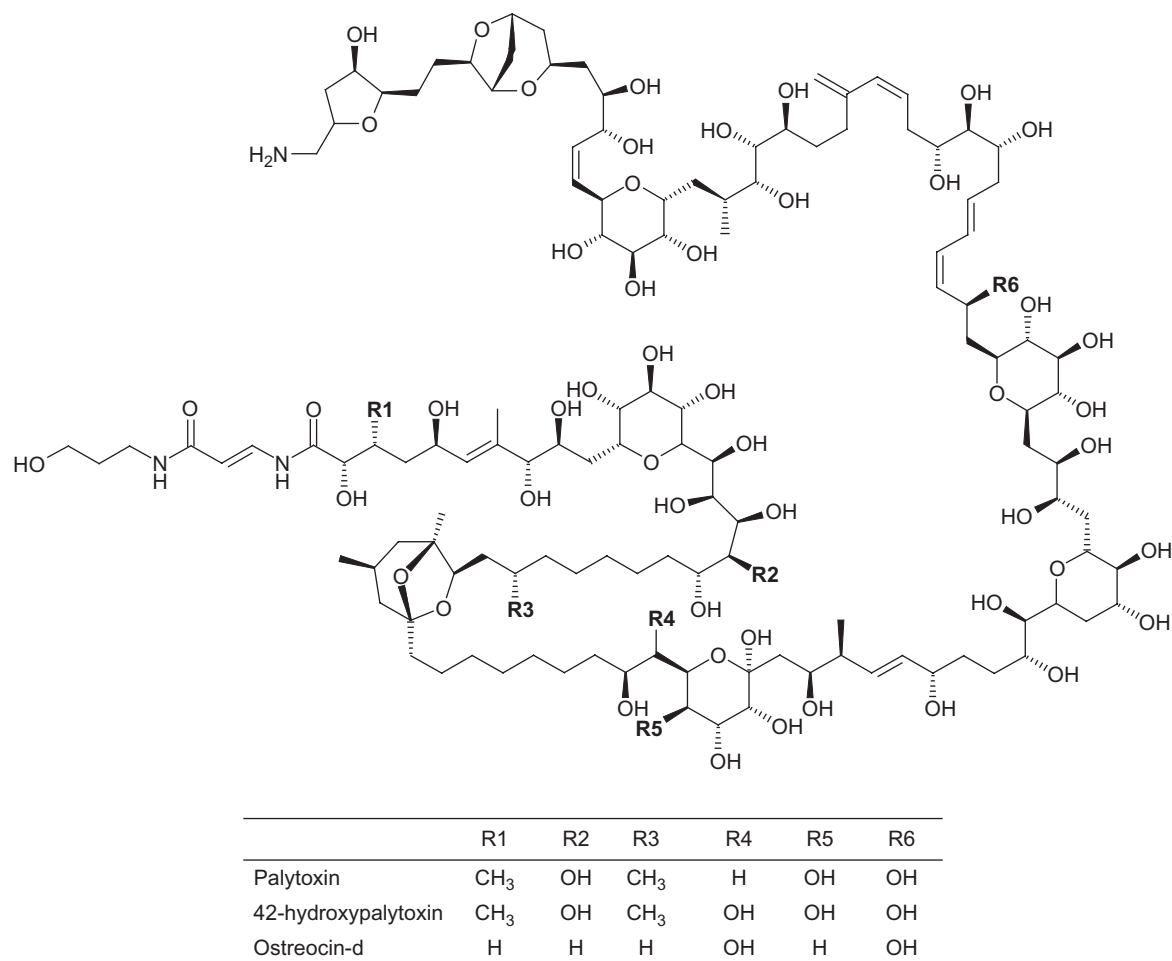


FIGURE 69.10 Chemical structure of palytoxin and two of its analogs (from Ciminiello *et al.*, 2011).

Ciminiello *et al.*, 2010). These species bloom in tropical and subtropical coastal waters, but their distribution has increased markedly in the past few years in temperate waters, such as the Mediterranean Sea (Rhodes, 2011).

A series of poisonings in humans have been associated with PLTXs by different exposure routes, although data confirming a direct relation between the illness and involvement of PLTXs are often lacking. The occurrence of toxic *Ostreopsis* species may result in the accumulation of PLTXs in edible marine organisms. Human illness and death arising from consumption of crabs and fish contaminated or suspected to be contaminated with PLTXs have been reported in tropical and subtropical areas. The most frequent symptoms include gastrointestinal ones, myalgia, muscle cramps, cardiac dysfunctions, respiratory problems, and cyanosis, with rhabdomyolysis the most commonly reported complication. Recent cases of human poisoning were associated with inhalation and/or cutaneous/systemic exposures after direct contact with aerosolized seawater during *Ostreopsis* blooms in Europe or through maintaining aquaria containing zoanthids. In these cases, the most common symptoms

were respiratory distress, rhinorrhea, cough, and fever. Dermatitis was also reported. Toxin identification and/or quantification are often incomplete or missing, and cases of poisoning are frequently ascribed to PLTXs on the basis of symptoms, anamnesis, and environmental/epidemiological data (i.e., zoanthid handling and ingestion of a particular fish or crab species) (Tubaro *et al.*, 2011a).

### Pharmacokinetics/toxicokinetics

No pharmacokinetic/toxicokinetic studies on PLTXs are available. An attempt to use Caco-2 cell lines to predict the PLTX absorption was hindered by its cytotoxicity (Pelin *et al.*, 2011).

### Mechanism of action

The main molecular target of PLTX seems to be Na<sup>+</sup>/K<sup>+</sup>-ATPase, a plasma membrane pump involved in the maintenance of transmembrane ionic gradients of animal



cells, which is essential for cell functions. Interaction of PLTX with  $\text{Na}^+/\text{K}^+$ -ATPase induces a change in the protein conformation that results in its conversion into a nonselective channel for monovalent cations. Therefore, the gates on the two sides of the membrane are simultaneously opened with consequent  $\text{Na}^+$  influx into the cells and  $\text{K}^+$  efflux, causing depolarization and triggering a series of adverse biological effects (Wu, 2009; Rossini and Bigiani, 2011).

## Toxicity

### Human toxicity

Human illnesses ascribed to PLTXs have occurred after seafood consumption but also after inhalation and cutaneous/systemic exposures to aerosolized seawater during *Ostreopsis* blooms or from handling zoanthids containing aquaria. Poisonings by oral exposure, with some fatalities, have occurred in tropical and subtropical areas. The most common signs and symptoms are gastrointestinal ones, myalgia, muscle cramps, cardiac dysfunctions, respiratory problems, and cyanosis, with frequent elevated serum levels of creatine phosphokinase and myoglobinuria raised by rhabdomyolysis. On the other hand, the most common signs after supposed inhalational and cutaneous/systemic exposures are respiratory distress, rhinorrhea, cough, fever, and a minor incidence of dermatitis. The actual toxicological potential of PLTXs has yet to be fully evaluated, as reported by Tubaro *et al.* (2011a). Despite the limited toxicological data on PLTXs, an oral acute reference dose (ARfD) of  $0.2\text{ }\mu\text{g/kg}$  (sum of PLTX and ostreocin-d) has been established using experimental toxicity data (EFSA, 2009a).

### Experimental toxicity

#### Single administration

The acute toxicity of PLTX to mammals is strongly dependent on the exposure route. PLTX is most toxic after parenteral administration. The rabbit, dog, rhesus monkey, and rat seem to be the most susceptible species to PLTX by i.v. injection, and the mouse seems to be the least susceptible, although wide variations in estimated  $\text{LD}_{50}$  values ( $0.025\text{--}0.53\text{ }\mu\text{g/kg}$ ) in mice are reported. This is also because different PLTX preparations (with varying purity) have been used, especially in the first studies. PLTX is less toxic i.p. than by i.v. injection, with  $\text{LD}_{50}$  values in mice ranging from  $0.31$  to  $1.5\text{ }\mu\text{g/kg}$ ; the i.p. toxicity in mice of the PLTX analog ostreocin-d (Figure 69.10) was lower (lethality  $>4\text{ }\mu\text{g/kg}$ ).  $\text{LD}_{50}$  values of PLTX by i.p. injection are similar to those after intramuscular and subcutaneous injections.

PLTXs are also highly toxic by intratracheal instillation: the lethal dose of PLTX and ostreocin-d was greater than 2 and  $11\text{--}13\text{ }\mu\text{g/kg}$ , respectively (Wiles *et al.*, 1974; Ito and Yasumoto, 2009). PLTX is much less toxic by the oral route, and in mice,  $\text{LD}_{50}$  values of 510 and  $767\text{ }\mu\text{g/kg}$  have been calculated (Munday, 2008a; Sosa *et al.*, 2009). Comparable lethality ( $\text{LD}_{50} = 651\text{ }\mu\text{g/kg}$ ) was estimated in mice for 42-hydroxyPLTX (Figure 69.10) (Tubaro *et al.*, 2011b), whereas no lethality was observed for ostreocin-d at  $300\text{ }\mu\text{g/kg}$  (Ito and Yasumoto, 2009). PLTX and ostreocin-d ( $200\text{ }\mu\text{g/kg}$ ) seem also to be toxic after sublingual administration to mice (Ito and Yasumoto, 2009).

Following PLTX i.p. injection in mice, adhesions were observed in the peritoneum, with ascites and small intestine dilation. At the histological level, the toxin induced single-cell necrosis in the hearts and necrosis of thymus and spleen lymphocytes. Bleeding, edema, and necrosis were recorded in the small intestine. Electron microscopy showed rounding of mitochondria and separation of organelles in myocytes, loss of microvilli in renal tubules, and vacuolation of pancreatic acinar cells (Terao *et al.*, 1992; Ito *et al.*, 1996). Ostreocin-d injection in mice caused erosion in the stomach and intestines (Ito and Yasumoto, 2009).

Intratracheal PLTX instillation in mice caused alveolar hemorrhage, pulmonary edema, gastrointestinal erosion, and glomerular atrophy. Similar lung injuries were observed in ostreocin-d-treated mice but showed slower progression and recovery than those from PLTX (Ito and Yasumoto, 2009).

After oral administration of PLTX, increased plasma levels of creatine phosphokinase, lactate dehydrogenase, and aspartate transaminase were recorded in mice. At the histological level, forestomach inflammation was observed in mice surviving up to 24 h after administration; other aspecific tissue alterations were noted in the liver and pancreas, whereas cardiac and skeletal muscle cells revealed only ultrastructural alterations visible by electron microscopy (Sosa *et al.*, 2009). Similar findings were noted in 42-hydroxyPLTX-treated mice, in which a plasma increase of potassium ions was also recorded (Tubaro *et al.*, 2011b). Application of PLTX to mouse ear skin induced irritation, and the dose causing erythema in 50% of mice was  $0.02\text{ }\mu\text{g/ear}$  (Fujiki *et al.*, 1986).

#### Repeated administration

After repeated i.p. injections of PLTX in mice ( $0.25\text{ }\mu\text{g/kg}$ , five times per week; total injections: 5, 10, 15, and 29), diarrhea and peritonitis were recorded after 29 doses (60% of mice). Necrosis of thymic and splenic lymphocytes was also observed. Moreover, decreased thymus weights and increased spleen weights were noted after 10–15 doses. These changes were reversible within 1 month after the 29th dose (Ito *et al.*, 1997).

#### Mutagenic and genotoxic activity

PLTX was negative in the Ames mutagenicity test using strains of *Salmonella typhimurium* with or without microsomal activation. Furthermore, it did not act as an initiator in the *in vitro* BALB/c 3T3 cell transformation assay or in mouse skin *in vivo* (Fujiki *et al.*, 1986; Munday, 2011).

#### Tumor-promoting activity

PLTX is a tumor promoter, as evidenced by two-stage carcinogenesis studies in mouse skin and in the *in vitro* BALB/c 3T3 cell transformation assay (Fujiki *et al.*, 1986; Munday, 2011).

#### Treatment

The treatment of poisonings implicating PLTX is mainly supportive and depends on the exposure route. After oral intake, treatments such as gastric lavage, forced diuresis therapy, artificial respiration, and fluids administration were applied but in some cases could not prevent fatalities. After inhalational and/or skin exposure, in some cases corticosteroids, nonsteroidal anti-inflammatory drugs, histamine antagonists, nebulized  $\beta$ -agonists, and/or oxygen therapy were administered to alleviate the symptoms, with recovery within a few hours or days (Tubaro *et al.*, 2011a).

#### Concluding remarks

PLTXs may contaminate edible marine organisms, with possible severe toxic effects in humans after seafood consumption. Adverse effects can also occur by inhalation and cutaneous/systemic exposures after contact with aerosolized seawater during *Ostreopsis* blooms and/or handling zoanthids containing aquaria. Episodes of poisoning have often been indirectly ascribed to PLTXs on the basis of symptoms, anamnesis, and environmental/epidemiological data. Thus, careful case reports are necessary to identify PLTXs as the causative agents of the poisoning and to unambiguously define the toxicological risk related to PLTX exposure. Despite the increasing occurrence of *Ostreopsis* species producing PLTXs in temperate coastal areas, there are no regulations on PLTXs in shellfish. Considering the lack of reliable quantitative data on PLTX toxicity in humans and the LOAEL of 200  $\mu\text{g}$  PLTX/kg for acute oral toxicity in mice as the reference point, an ARfD of 0.2  $\mu\text{g}/\text{kg}$ , applied to the sum of PLTX and ostreocin-d, has been established by the European Food Safety Authority to propose 30  $\mu\text{g}/\text{kg}$  as tolerance limit in shellfish meat (EFSA, 2009a).

To detect PLTXs in shellfish, cell-based assays such as cytotoxicity assays on neuroblastoma cells and hemolysis assay have also been developed but not standardized.

High-performance liquid mass spectrometry methods and LC-MS/MS methods are promising tools for PLTX detection. However, their optimization and validation, as well as the development of certified reference materials and standards, are necessary (EFSA, 2009a; Ciminiello *et al.*, 2011).

## OKADAIC ACID AND ITS DERIVATIVES

### Background

Okadaic acid (OA) and its derivatives provoke the human illness called diarrhetic shellfish poisoning (DSP). DSP symptoms are mainly gastrointestinal distress, diarrhea, nausea, vomiting, and abdominal cramps, occurring between 30 min and several hours after contaminated shellfish consumption. It is not a fatal illness, and complete recovery occurs within 3 days, generally without any treatment. Nevertheless, DSP is an important cause of morbidity worldwide, causing serious problems for the shellfish industry and regulators. DSP toxins are lipid-soluble and heat-stable polyethers, including OA and its derivatives, dinophysins (DTXs), the most frequent being DTX-1, DTX-2, and DTX-3 (Figure 69.11). OA, the main DSP toxin originally isolated from *Halichondria okadae* sponge, is produced by dinoflagellates of the genus *Dinophysis*, similarly to DTX-1 and DTX-2. These compounds are also produced by cultured dinoflagellates of the *Prorocentrum* genus, but these species were sporadically detected in phytoplankton during DSP outbreaks. DTX-3, a mixture of OA, DTX-1, and/or DTX-2 esterified with a fatty acid generally at the 7-hydroxy group, is believed to be a metabolic product of shellfish rather than algal metabolites (Tubaro *et al.*, 2008a). The first documented dinoflagellate species involved in DSP was *D. fortii* in Japan and later in other areas. Other species, such as *D. acuminata*, *D. acuta*, *D. caudata*, *D. mitra*, *D. norvegica*, *D. rotundata*, *D. tripos*, *D. sacculus*, and *D. trunculus*, have also been reported as sources of DSP toxins. Toxin production may vary considerably among these species as well as among regional and seasonal morphotypes in one species (Hallegraeff, 2003). Several bivalves, such as mussels, scallops, oysters, and clams, by filtering the seawater and the toxic phytoplankton, can accumulate the toxins and cause human poisoning after their ingestion.

DSP cases have been reported in many different areas of the world, but most cases have been reported in Japan, Europe, and, to a lesser extent, North and South America, Thailand, Australia, and New Zealand (Hallegraeff, 2003; Fux *et al.*, 2011). One major DSP outbreak, involving more than 300 people, occurred in Italy in September 2010. In 2011, the first documented

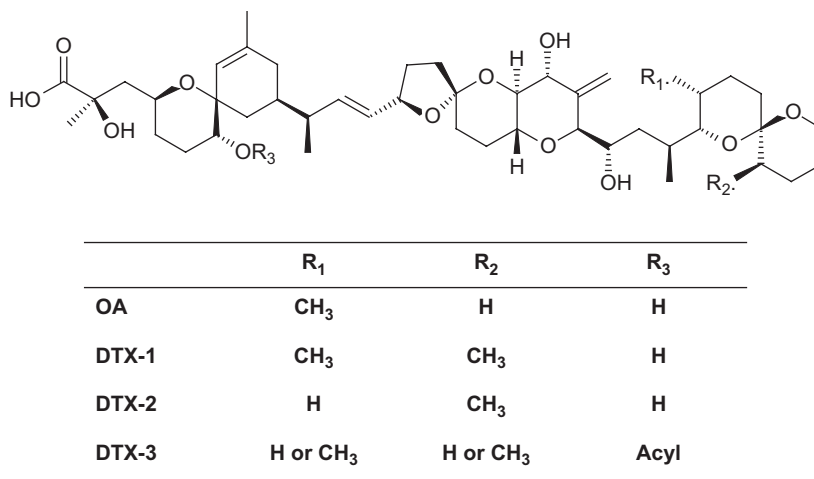


FIGURE 69.11 Chemical structure of okadaic acid (OA) and dinophys toxin (DTX)-1, -2, and -3 (from Quilliam, 2003).

outbreaks of DSP in North America occurred in waters of the Pacific Northwest: more than 60 cases were reported from shellfish harvested at Salt Spring Island, British Columbia, Canada, and farther south, 3 cases were reported from shellfish of Sequim Bay, Washington (NOAA, 2011). LC-MS/MS analysis revealed the presence of DTX-1 and its fatty acid esters. DSP cases, or at least the presence of algae-producing OAs, appear to be increasing, but this perception may also be influenced by improved knowledge of the disease and surveillance programs.

### Pharmacokinetics/toxicokinetics

Some toxicokinetic studies on OA have been performed in mice. Twenty-four hours after single oral [<sup>3</sup>H]OA administration to mice (50 or 90 µg/kg), the toxin was detected in all organs and fluids, including skin, and was most concentrated in urine, intestinal contents, and intestine. The slow OA elimination suggested an enterohepatic circulation (Matias *et al.*, 1999). A study by Ito *et al.* (2002a) revealed that after wider oral doses of OA in mice (75–250 µg/kg), the toxin was quickly absorbed from the small intestine, mainly from jejunum, reaching the liver within 5 min. The distribution of the toxin, evaluated by an immunostaining method, involved the whole body, being detected in lungs, liver, heart, kidneys, and intestine, in which it was still detectable after 2 weeks. Excretion from kidneys and intestine began 5 min after administration, and elimination through feces continued for 4 weeks. Another immunohistochemical study after acute oral OA administration in mice (115 and 230 µg/kg) showed the presence of low amounts of the toxin in the liver 24 and 48 h

later, respectively; the toxin was detected in the duodenum and ileum (localized in the mucosal secretions of the goblet cells) but not in the colon secretions (Le Hégarat *et al.*, 2006). Thus, after acute oral administration in mice, OA seems to be distributed in the following rank order: intestinal content > urine > feces > intestine tissue > lung > liver > stomach > kidney > blood (Toyofuku, 2006).

After i.p. injection of [<sup>3</sup>H]OA to mice (14 µCi/0.2 mL), most of the radioactivity (33%) was detected in the gastrointestinal contents 3 h later, whereas 5% was measured after 19 h. In the liver, 27 and 16% of the radioactivity was detected after 3 and 19 h, respectively, suggesting a hepatobiliary circulation (Fujiki and Suganuma, 1993; Nishiwaki *et al.*, 1994).

After intramuscular injection of [<sup>3</sup>H]OA to mice (25 µg/kg), the toxin was detected in bile and intestinal contents 1 h later, and its elimination pattern showed biliary excretion and enterohepatic circulation. OA transplacental passage in mice was also observed (Matias and Creppy, 1996a,b).

A poisoning episode in humans due to ingestion of DTX-3-contaminated mussels gave evidence for a biotransformation into DTX-1, which was the only DSP toxin detected in the feces (García *et al.*, 2005).

An *in vitro* study using Caco-2 cells as a model of human intestinal barrier showed that low-level exposure of the cell monolayer to OA (20–200 nM) allows only limited passage of the toxin from the “luminal” to the “blood side,” but a significant efflux was observed, suggesting an active transport mechanism for OA elimination (Ehlers *et al.*, 2011). Another *in vitro* study showed OA biotransformation to oxygenated metabolites by two liver P450 cytochromes – a process that does not seem to detoxify OA (Guo *et al.*, 2010).

## Mechanism of action

OA and DTX-1 inhibit protein phosphatases, mainly protein phosphatases 1 and 2A, which are two enzymes that dephosphorylate serine/threonine residues of eukaryotic cell proteins (Bialojan and Takai, 1988). As a consequence, a rapid increase in phosphorylated proteins occurs in cells. The diarrhea and the degenerative changes in absorptive epithelium of small intestine induced by these toxins have been attributed to an increased level of the phosphorylated proteins controlling ions secretion in intestinal cells and of cytoskeletal and/or junctional elements regulating the permeability to solutes: these events result in a passive loss of fluids (Domínguez *et al.*, 2010). Inhibition of protein phosphatases is also involved in other effects, including the tumor-promoting properties of these toxins (Fujiki and Suganuma, 1993). A free carboxyl group in the DSP molecule is essential mechanistically because methyl and diol esters do not show inhibition (Mountfort *et al.*, 2001).

## Toxicity

### Human toxicity

DSP symptoms are mainly diarrhea, nausea, vomiting, and abdominal pain. Although the intoxication can be highly debilitating for some days, no human mortalities from DSP have been reported. Recovery is usually complete in 3 days, despite the pharmacological treatment. A LOAEL of 0.8 µg OA equivalents/kg has been estimated, deriving an ARfD of 0.3 µg OA/kg (EFSA, 2008a).

### Experimental toxicity

#### Single administration

After single i.p. injection in mice, the LD<sub>50</sub> of OA ranged from 192 to 225 µg/kg (Tubaro *et al.*, 2008a). An LD<sub>50</sub> of 352 µg/kg was determined for DTX-2, and a relative potency of 0.6 was estimated compared to that of OA, which was ascribed to the axial 35-methyl group of DTX-2 that would give rise to lower affinity for PP2A (Aune *et al.*, 2007; Huhn *et al.*, 2009). Intraperitoneal injection of OA or DTX-1 (≥200 and 375 µg/kg) in rodents (mice and rats) induced damages in the intestinal mucosa, particularly the duodenum and upper part of the jejunum, within 15 min. The injuries can be divided into three consecutive stages: increased capillary permeability and extravasation of serum into the lamina propria of villi, degeneration of absorptive epithelium of villi, and desquamation of the degenerated epithelium from the lamina propria. At sublethal doses, these alterations are reversible, and the recovery process, observed already after 2 h, is complete within 24 h (Terao *et al.*, 1986, 1993; Ito and Terao, 1994). Morphological changes

induced by DTX-3 are less pronounced and consist only of dilation of the cisternae of the Golgi apparatus and the presence of vesicles in the cytoplasm of the absorptive epithelium (Terao *et al.*, 1993). Intraperitoneal injection of OA, DTX-1, or DTX-3 in rodents also induces liver damage, with vacuolization and/or necrosis of hepatocytes (Terao *et al.*, 1993; Ito and Terao, 1994; Aune *et al.*, 1998; Tubaro *et al.*, 2003).

OA-induced liver damage also occurs after i.v. injection in rats, with congestion of blood in the liver and dissolution of hepatic bile canalicular actin sheaths (Berven *et al.*, 2001).

OA, DTX-1, and DTX-3 are less toxic orally than intraperitoneally. By the oral route, the LD<sub>50</sub> of OA in mice ranges between 1 and 2 mg/kg (Tubaro *et al.*, 2003). In addition to diarrhea, the signs of toxicity are similar to those observed after i.p. injection. DTX-1 and DTX-3 also cause the degeneration of surface cells of the gastric mucosa (Terao *et al.*, 1993; Ito and Terao, 1994; Ito *et al.*, 2000; Berven *et al.*, 2001).

#### Repeated administration

Daily repeated oral OA administration in mice (1 mg/kg/day for 7 days) induced diarrhea, body weight loss, reduced food consumption, and death of two of five mice after 5 days. Toxic effects are noted in forestomach and liver, whereas ultrastructural changes are seen in cardiomyocytes (mitochondria and fibers) (Tubaro *et al.*, 2004).

#### Mutagenic and genotoxic activity

Although OA is not mutagenic in *S. typhimurium* in the absence or presence of metabolic activation, it is mutagenic in various eukaryotic cells *in vitro* (Aune and Yndestad, 1993; Fessard *et al.*, 1996). No genotoxicity data are available for DTX-2 and DTX-3. Genotoxic effects of OA, such as micronuclei formation, mitotic arrest, and polyploidy, were observed in human Caco-2 cells (Le Hégarat *et al.*, 2006). OA is not reported on any of the lists of the International Agency of Research on Cancer.

#### Tumor-promoting activity

OA and DTX-1 are tumor promoters, as evidenced by two-stage carcinogenesis studies on mouse skin (Fujiki and Suganuma, 1993) and rat glandular stomach (Suganuma *et al.*, 1992).

## Treatment

The treatment of the diarrheic poisoning is symptomatic and supportive with regard to short-term diarrhea and loss of fluids and electrolytes. In general, hospitalization is not necessary. Other diarrheic illnesses associated with



shellfish consumption, such as bacterial and viral contamination, should be ruled out on the basis of anamnesis of the patients (Aune and Yndestad, 1993).

### Concluding remarks

OA and DTXs are involved in DSP and possess tumor-promotion activity through their inhibition of protein phosphatases. DSP risk is managed by monitoring seafood for toxicity: frequency of sampling should ensure that contamination does not rise to dangerous levels in temporal or spatial gaps between sampling times or locations. Monitoring programs should also assess the toxin-producing phytoplankton organisms in the water column. When this is accomplished in real time, a potentially toxic bloom can be detected earlier than chemical analysis can be applied to detect shellfish contamination. An example is provided by the first instance in which OA was confirmed in U.S. shellfish: although OA was later detected in Texas shellfish by LC-MS/MS (Deeds *et al.*, 2010), the harvest had already been closed due to prior detection of the (*Dinophysis*) bloom by a remote instrument, the FlowCytobot (Campbell *et al.*, 2010).

Various methods have been developed for analysis of DSP toxins, including *in vivo* bioassays and *in vitro* biochemical or biological assays, as well as chemical methods of analysis. The MBA is the accepted method for DSP toxins determination in shellfish because it can detect all DSP toxins, although other lipophilic compounds can interfere. It evaluates the survival time of mice injected i.p. with a suspension of a shellfish extract usually over a 24-h observation period (Fernández *et al.*, 2003). Immunoassays include enzyme-linked assays (ELISA kits are commercially available) or radioactivity-based immunoassays and also lateral flow immunochromatography test kits, which are threshold based, use visual endpoints, and are now commercialized. Protein phosphatase 2A inhibition assays are particularly sensitive *in vitro* functional assays, being based on the mechanism of OA action (Cembella *et al.*, 2003), and have been commercialized in test kit formats. Chemical methods of analysis include HPLC, capillary electrophoresis, and LC-MS techniques (Quilliam, 2003). For the functional, ELISA, and instrumental methods, the results should include the toxins release by hydrolysis of their esters. The maximum tolerated level of these toxins was set at 160 µg OA equivalents/kg of shellfish meat (European Commission, 2002).

Regarding the control of DSP toxins, their regulation in most countries is based on the negative results of shellfish samples by the MBA. The European Union is phasing out the MBA (European Commission, 2011), which will be replaced by an LC-MS/MS method that has been interlaboratory validated at the EU Reference

Laboratory for Marine Biotoxins (Villar-González *et al.*, 2011). Alternatives or complementary methods can be used when properly validated; however, there are inherent advantages in using LC-MS/MS to monitor lipophilic toxins because several classes of toxins addressed in regulations can be detected at once.

## AZASPIRACIDS

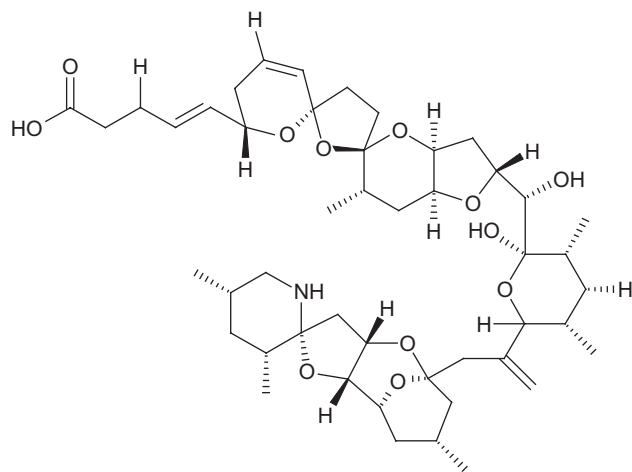
### Background

Azaspiracid poisoning (AZP) is a relatively recent development, first reported in 1995 in the Netherlands when eight people became ill following ingestion of blue mussels from Ireland. The victims developed symptoms very similar to those of DSP, despite the low content of OA and its derivatives. Subsequently, the azaspiracid toxin group was discovered. The structural elucidation of the first AZP toxin, azaspiracid1 (AZA1), showed its polyether backbone with a unique spiral ring assembly, a cyclic amine, and a carboxylic acid (Figure 69.12). More than 30 analogs have been identified, but AZA1, AZA2, and AZA3 are the most important ones based on occurrence and toxicity (Twiner *et al.*, 2008; EFSA, 2008b).

AZAs, first detected in blue mussels (*M. edulis*), have been found in other bivalves, including other *Mytilus* species, oysters (*Crassostrea gigas* and *Ostrea edulis*), scallops (*Pecten maximus* and *Argopecten purpuratus*), clams (*Tapes philippinarum*, *Ensis siliqua*, and *Donax* spp.), and cockles (*C. edule*), as well as in crustaceans (*Cancer pagurus*) (Twiner *et al.*, 2008; López-Rivera *et al.*, 2010). Reports of AZA poisonings and/or contaminated shellfish have been documented from several European countries, including Ireland, the United Kingdom, Norway, the Netherlands, France, Spain, and Italy. The source of AZAs has been identified as the dinoflagellate *Azodinium spinosum*. Although AZAs have been identified mainly in western Europe, they have recently been detected in northwestern Africa, eastern Canada, and South America. AZP may be widespread but generally under-reported because its gastrointestinal symptoms are similar to those of DSP or of bacterial enterotoxin poisoning (Furey *et al.*, 2010).

### Pharmacokinetics/toxicokinetics

Aasen *et al.* (2010, 2011) observed absorption of AZA1 after acute oral administration to mice (100–300 µg/kg). A dose-dependent increase in the toxin concentration in tissues was observed, and after 24 h, the highest levels of AZA1 were found in the gastrointestinal tissues (stomach > duodenum > jejunum > ileum > colon); in other



**FIGURE 69.12** Chemical structure of azaspiracid-1 (from James *et al.*, 2004).

organs, the order of ranking was lungs > heart > liver, and only trace amounts were detected in the brain. After 7 days, the levels of AZA1 had markedly decreased in all organs except the kidneys. The amount of toxin was the highest in the liver, followed by kidneys, lungs, spleen, and heart. After 24h, the total amount of toxin in the internal organs was approximately 2% of the administered doses.

### Mechanism of action

The mechanism of action of AZAs is unclear. However, several effects on *in vitro* cell cultures have been reported for AZA1, including cytoskeletal alterations and decreased F-actin pools, with irreversible effects after toxin withdrawal; alteration of tight junction proteins and E-cadherin degradation; activation/apoptosis; membrane cholesterol reduction; cytosolic  $\text{Ca}^{2+}$  and cAMP level modulation; and alteration of expression of different cellular proteins and energy metabolism as well as neuronal cells signaling (Ryan *et al.*, 2008; Vilariño, 2008; Furey *et al.*, 2010). The AZA-induced F-actin and cytoskeletal alterations and tight junction protein changes could be responsible for some of the *in vivo* toxic effects, such as the gastrointestinal toxicity and diarrheic activity. A possible factor contributing to increased fluid secretion in AZA-induced diarrhea seems to be the increased paracellular permeability of intestinal epithelium, as suggested by *in vitro* findings in human intestinal Caco-2 cells. In fact, alterations in the levels and localization of tight junction proteins, involved in the regulation of intestinal barrier function, were observed and correlated with the increased paracellular permeability (Ryan *et al.*, 2008; James *et al.*, 2010).

## Toxicity

### Human toxicity

The human symptoms include nausea, vomiting, severe diarrhea, and stomach cramps (James *et al.*, 2010). A LOAEL of 1.9  $\mu\text{g}$  AZA1 equivalents/kg was estimated, and an ARfD of 0.2  $\mu\text{g}$  AZA1/kg was established (EFSA, 2008b).

### Experimental toxicity

#### Single administration

By i.p. injection, the lethal dose of AZA1 in mice has been determined to be 200  $\mu\text{g}/\text{kg}$ ; AZA2 and AZA3 are more toxic (110 and 140  $\mu\text{g}/\text{kg}$ , respectively), contrary to AZA4 (470  $\mu\text{g}/\text{kg}$ ) and AZA5 (<1 mg/kg). Although diarrhea is the main symptom in humans, AZAs did not cause diarrhea by i.p. injection: before death, mice showed progressive paralysis of the limbs, dyspnea, and convulsions (James *et al.*, 2004; Twiner *et al.*, 2008).

After oral administration of AZA1 ( $\geq 300 \mu\text{g}/\text{kg}$ ) to mice, necrosis in the lamina propria of the small intestine and in thymus, spleen, and Peyer's patches was observed (necrotic T and B lymphocytes). Fatty changes in the liver were also observed. The authors hypothesized that the oral toxicity is 2.5 times higher than that after i.p. injection, but no data on  $\text{LD}_{50}$ s are available (Ito *et al.*, 2000). Following sublethal dosing of AZA1 (100–300  $\mu\text{g}/\text{kg}$ ) to mice, pathological changes were only detected in duodenum after 24h (mild cellular detachment in the tips of the villi, expansion of the crypts and necrotic/apoptotic changes in lamina propria, and neutrophil infiltration), which were recovered after 7 days (Aasen *et al.*, 2010). No data on oral toxicity are available for AZA analogs.

#### Repeated administration

After repeated oral intake of sublethal doses of AZA1 (250–450  $\mu\text{g}/\text{kg}$ ), death in some mice occurred, and serious gastrointestinal, pulmonary, and hepatic effects that persisted for a prolonged period in survived mice were observed. Repeated oral administration of AZA1 once or twice a week (20–50  $\mu\text{g}/\text{kg}$  for 10–20 weeks) caused interstitial pneumonia, shortening of intestinal villi, as well as death of some mice. Lung tumors developed in 4/20 mice treated with 20 or 50  $\mu\text{g}$  AZA1/kg, but further studies are needed to confirm the carcinogenicity of this toxin (Ito *et al.*, 2002b). No genotoxicity data are available, and no definitive conclusions regarding relevance to humans can be drawn (EFSA, 2008b; Twiner *et al.*, 2008).

## Treatment

As for DSP, a specific antidote is not available for AZP. The treatment is only symptomatic and supportive.

## Concluding remarks

The maximum overall level of azaspiracid was set by the European Union at 160 µg of AZA1 equivalents/kg of edible meat (European Commission, 2002). Mouse or rat bioassay can detect AZAs with a limit of detection of approximately 160 µg/kg, but other lipophilic toxins can potentially interfere. As with the okadaic acids, European use of the MBA in detecting the azaspiracids is being gradually phased out, to be replaced by the same LC-MS/MS methodology approved for OA toxins and other lipophilic marine toxins of concern in Europe (Villar-González *et al.*, 2011). Alternative or complementary methods may possibly be used after their international validation (European Commission, 2011).

## OTHER LIPOPHILIC TOXINS

### YESSOTOXINS

#### Background

Yessotoxin (YTX) (Figure 69.13) is a ladder-shaped polycyclic ether that was first isolated from the scallop *Patinopecten yessoensis*. YTX and its analogs were initially included in the DSP group, being detected with OA

and other lipophilic toxins during the shellfish extraction for DSP bioassay. Subsequently, YTXs have been classified and regulated separately because they do not induce diarrhea. More than 30 YTX analogs have been isolated from shellfish or microalgae, and the existence of approximately 100 analogs has been reported to date. No human toxicity has been ascribed to YTXs, but many YTXs are highly toxic to mice intraperitoneally (Tubaro *et al.*, 2010).

YTXs are produced by dinoflagellates such as *Protoceratium reticulatum* (= *Gonyaulax grindley*), *Lingulodinium polyedrum* (= *Gonyaulax polyedra*), and *Gonyaulax spinifera*. Because these algal species are common in coastal waters of many regions, YTXs may occur worldwide, and during their filter-feeding activity, edible shellfish can concentrate these compounds (Paz *et al.*, 2008).

#### Pharmacokinetics/toxicokinetics

Twenty-four hours after acute oral YTX administration in mice (1 and 5 mg/kg), the highest toxin levels were found in ileum, followed by colon, jejunum and duodenum, kidneys, spleen, heart, and blood (Aasen *et al.*, 2011). Twenty-four hours after the last gavage to mice (1 mg YTX/kg/day for 7 days), Tubaro *et al.* (2008b) detected a mean YTX blood concentration of approximately 6 ng/ml (5 nM) by an ELISA method. No data on the metabolism of YTXs are available.

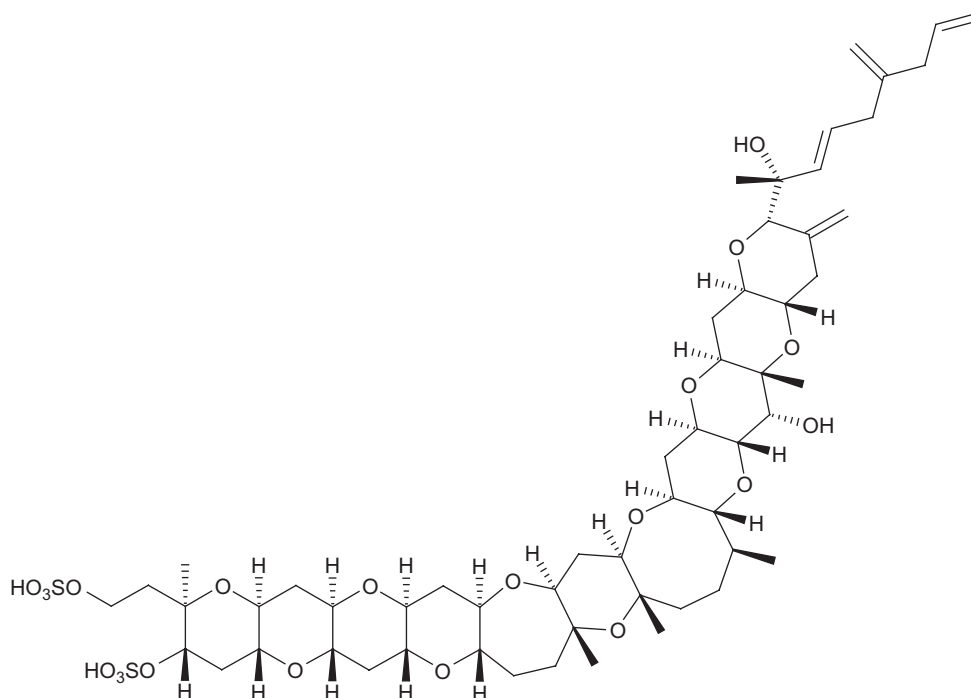


FIGURE 69.13 Chemical structure of yessotoxin (from Quilliam, 2003).

## Mechanism of action

Although low concentrations of YTX affect many systems *in vitro*, some of these effects are not confirmed *in vivo* after *per os* exposure and the precise mechanism of action of YTXs is not yet known. It is known that YTX is a cytotoxic against several cell lines, affecting a variety of cellular activities. YTX has been shown to induce apoptosis-activating caspases, to open the mitochondrial permeability transition pore, to impair protein disposal and phagocytic activity, to affect cytoskeletal components, and to modulate intracellular  $\text{Ca}^{2+}$  and cAMP levels. YTX has also been shown to inhibit cardiomyocyte beating frequency (Tubaro *et al.*, 2010).

## Toxicity

### Human toxicity

There are no reports of human poisoning by YTXs. A dose of 5mg/kg has been adopted as the most robust no-observed-adverse-effect level for acute cardiotoxicity of YTX on the basis of light microscopy findings in mice; an ARfD of 25µg YTX equivalents/kg has been established (EFSA, 2008c).

### Experimental toxicity

Only YTX, de-sulfo-YTX, homo-YTX, and 45-OH-homoYTX have been submitted to actual toxicological studies.

#### Single administration

After i.p. injection in mice, LD<sub>50</sub> values ranging from 100 and 750µg/kg were reported for YTX and homoYTX, whereas 45-OH-homoYTX was not lethal at 750µg/kg (Tubaro *et al.*, 2010). Light microscopy revealed only slight intracellular edema in the heart of animals treated with 750 and 1000µg/kg (Aune *et al.*, 2002). Electron microscopy revealed swelling of some cardiomyocytes and rounded mitochondria (Terao *et al.*, 1990; Aune *et al.*, 2002; Tubaro *et al.*, 2003). Damages to cerebellar Purkinje cells after i.p. injection of YTX (420µg/kg) were also observed, but these were not confirmed by oral exposure (Hungerford, 2006).

After oral administration, YTXs are much less toxic. No lethality or signs of toxicity were observed in mice after acute administration of YTX (0.5–10mg/kg). Only ultrastructural changes were noted in myocardium, similar to those described after i.p. injection (Terao *et al.*, 1990; Aune *et al.*, 2002; Tubaro *et al.*, 2003). Although YTX induces apoptosis *in vitro* in various cell lines, no apoptotic changes were noted in the myocardium of mice by *in situ* TUNEL staining (Tubaro *et al.*, 2003). A study found that the oral effects of YTX (1 and 5mg/kg) are

not enhanced by a sublethal (200µg/kg) dose of AZA1 (Aasen *et al.*, 2011).

#### Repeated administration

Following repeated oral administration for 7 days, no deaths, changes in behavior or growth, or macroscopic abnormalities were observed for YTX (1 and 2mg/kg/day), homoYTX, and 45-OH-homoYTX (1mg/kg/day). Only ultrastructural changes in cardiomyocytes (rounded mitochondria and alterations of cell boundary) were observed (Tubaro *et al.*, 2004). These ultrastructural changes were also noted 30 days after the last YTX administration (1mg/kg/day) but seemed to be recovered within 90 days (Tubaro *et al.*, 2008b). In another study, mice were exposed to YTX seven times during 21 days (1–5mg/kg) and sacrificed 3 days after the last treatment: by electron microscopy, vacuoles were seen in the myocardium at the highest toxin dose (Espenes *et al.*, 2006).

## Concluding remarks

Due to the lack of evidence of adverse effects in humans and the significant reduction in potency after oral administration compared to i.p. injection in mice, a regulatory level of 1mg YTX equivalents/kg shellfish has been established in some countries. For regulatory purposes, LC-MS/MS has been adopted in the European Union as the reference method for YTX detection in shellfish, but alternative or complementary methods can be used (European Commission, 2011).

## PECTENOTOXINS

### Background

Pectenotoxins (PTXs) are hepatotoxic compounds that were initially classified as DSP toxins because DSP toxins are often detected in shellfish and phytoplankton together with these compounds. Nevertheless, despite the fact that a PTX derivative was associated with severe diarrheic illnesses in Australia, the actual diarrhogenic potential of PTXs and the actual health threat to consumers of contaminated mussels are unclear (EFSA, 2009b; Dominguez *et al.*, 2010).

PTXs are polyether macrolides containing a spiroketal group, three substituted oxolanes, a bicyclic ketal, and a six-membered cyclic hemiketal. PTXs have been detected in shellfish and/or phytoplankton from many areas of the world, including Japan, Australia, New Zealand, and Europe. More than 20 PTX analogs (Figure 69.14) have been identified in phytoplankton or shellfish, and several of them derive from a parent PTX that is metabolized



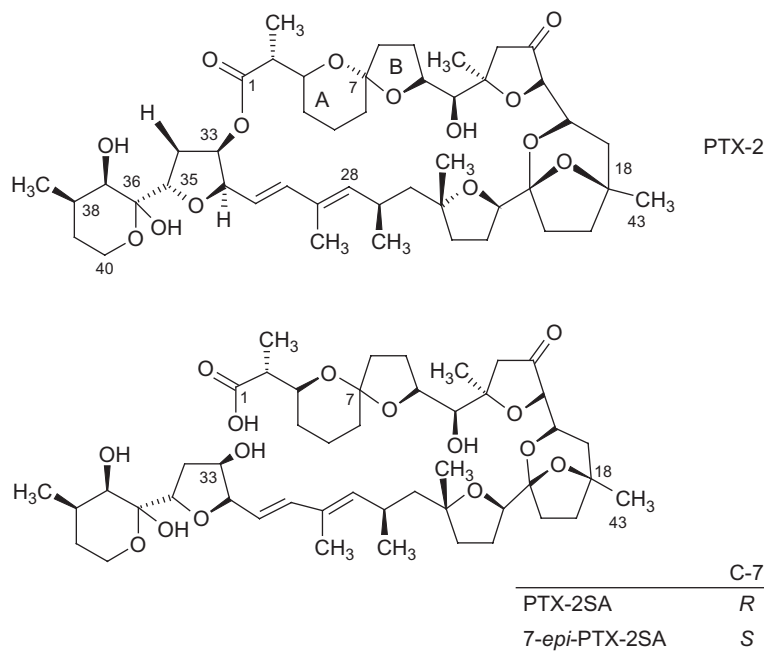


FIGURE 69.14 Chemical structure of pectenotoxin-2 (PTX-2) and its seco acids (from Draisci *et al.*, 2000).

within the shellfish. The most commonly found PTX in algae is PTX-2, produced by dinoflagellates of the genus *Dinophysis*. PTXs are accumulated in the digestive glands of filter-feeding bivalves, such as scallops (*Patinopecten yessoensis*), green-shell mussels (*Perna canaliculus*), blue mussels (*M. galloprovincialis* and *M. edulis*), cockles (*C. edule*), and pipis (*Donax delatoides*), after filtration of *Dinophysis* cells (Munday, 2008b; Suzuki, 2008; Dominguez *et al.*, 2010). These organisms can transfer the toxins to humans after their consumption.

### Pharmacokinetics/toxicokinetics

The distribution of PTX-2 and PTX-2 seco acid was studied in mice by oral and i.p. routes. After oral intake, both toxins were detected mainly in the gastrointestinal tract content and/or feces, only a small portion of toxins were found in the gastrointestinal tissue, and an extremely low amount of PTX-2 seco acid was found in the liver. After i.p. injection, the distribution of the toxins was similar, with a slightly higher presence in the liver and detectable levels in blood and kidney. Moreover, a considerable amount of PTX-2 was converted to PTX-2 seco acid or 7-*epi*-PTX-2 seco acid. Thus, PTXs do not seem to be readily absorbed after oral intake (Munday, 2008b).

### Mechanism of action

The mechanism of hepatotoxic action of PTXs has to be clarified. PTXs are cytotoxic against several cancer cell

lines, and PTX-1 and PTX-2 have been shown to induce apoptotic effects. *In vitro* studies revealed a disruption of actin cytoskeleton by PTX-1, PTX-2, and PTX-6. Furthermore, PTX-6 modified cAMP levels in human lymphocytes (Leira *et al.*, 2002; Espiña and Rubiolo, 2008; Dominguez *et al.*, 2010).

## Toxicity

### Human toxicity

PTXs have been associated with severe diarrheic illness in Australia. However, although the shellfish involved in the poisoning contained PTX-2 seco acid, the gastrointestinal effects were later attributed to OA esters. Consequently, no evidence of gastrointestinal or other adverse effects in humans from PTXs is available. For intestinal toxicity of PTX-2 observed in mice, a LOAEL of 250 µg/kg was considered and an ARfD of 0.8 µg/kg was established (EFSA, 2009b).

### Experimental toxicity

#### Single administration

After i.p. injection in mice, LD<sub>50</sub> values of PTXs were determined to be 250 µg/kg (PTX-1 and PTX-11), 219–260 µg/kg (PTX-2), 350 µg/kg (PTX-3), 770 µg/kg (PTX-4), and 500 µg/kg (PTX-6), whereas the LD<sub>50</sub> of PTX-7, PTX-8, PTX-9, PTX-2 seco acid, and 7-*epi*-PTX-2 seco acid was greater than 5 mg/kg. Thus, it seems that the oxidation of substituent in C-18, occurring in the

digestive glands of shellfish, reduces the toxicity of PTXs (Draisci *et al.*, 2000; Dominguez *et al.*, 2010).

A hepatotoxic effect was observed in suckling mice for PTX-1 as well as for PTX-1, PTX-2, PTX-6 in adult mice and rats. None of the toxins caused diarrhea, and no damage at the intestinal level was observed in suckling mice after i.p. injection of up to 1 mg PTX-1/kg. On the other hand, PTX-1 and -2 induced an increased permeability of capillaries in the digestive tract and ascites and increased fluid accumulation in the thorax and pericardium of adult mice and rats at a dose of 375 µg/kg (Terao *et al.*, 1986, 1993). Hepatotoxic effects were also observed for PTX-6, which caused severe bleeding in the liver, whereas PTX-2 caused congestion under the liver capsule from circulatory disorder (Ito *et al.*, 2008).

Although some data on PTX toxicity after oral administration are controversial, Terao *et al.* (1993) observed that PTX-1 and PTX-2 (750 µg/kg) did not induce changes at the intestinal level of mice and rats. No lethality and no diarrhetic effects were evidenced for PTX-2, PTX-2 seco acid, and PTX-6 in mice at a dose of 5 mg/kg (Miles *et al.*, 2004; Ito *et al.*, 2008). After PTX-6 oral administration to rats (2 mg/kg), the middle-lower small intestine was eroded at villi by edema. Moreover, no synergistic toxicity of PTX-6 with OA or PTX-2 after oral administration to mice was observed (Ito *et al.*, 2008).

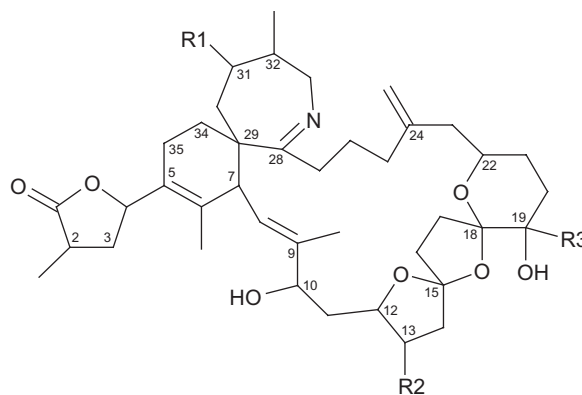
## Concluding remarks

The European Union has not set a maximum overall level for PTXs alone in shellfish because the established level of 160 µg/kg of edible parts is referred to as the sum of PTXs and diarrhetic toxins of the OA group (European Commission, 2002). MBA can detect PTXs but not the seco acids. The presence of other lipophilic toxins, such as AZAs and DSP toxins, can be detected by the mouse bioassay. Because PTXs are synthesized by the same microalgae-producing OA and its derivatives, it is important to distinguish the two toxin groups. LC-MS/MS has been adopted in the European Union as the reference method for detection of at least PTX-1 and PTX-2 in shellfish, but alternative or complementary methods, such as immunochemical assays, can be used if properly validated (European Commission, 2011).

## CYCLIC IMINE TOXINS

### Background

In recent years, a group of macrocyclic compounds with imine- and spiro-linked moieties have been discovered through their rapid lethality in mice by the mouse



Spirolide	R1	R2	R3
A	H	CH <sub>3</sub>	CH <sub>3</sub>
B	H	CH <sub>3</sub>	CH <sub>3</sub>
C	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
D	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>

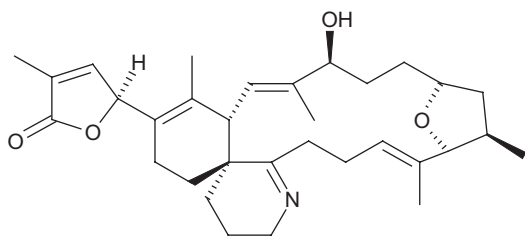
FIGURE 69.15 Chemical structure of spirolides A–D (from Ciminiello and Fattorusso, 2004).

bioassay used for detection of lipophilic shellfish toxins. These toxins include spirolides, gymnodimines, pinnatoxins, pteriatoxins, and prorocentrolides, and their threat to human health is unclear (Cembella and Krock, 2008; Munday, 2008c).

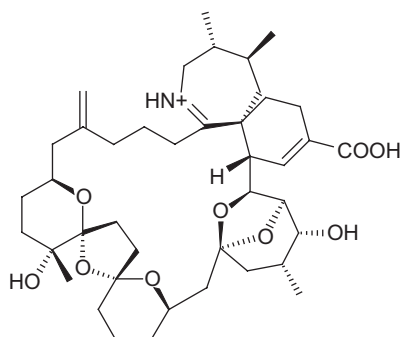
Several spirolides, possessing a spiro-linked tri-cyclic ether structure and an imine or amine function, have been identified in shellfish and phytoplankton: spirolides A–G and their derivatives, among which spirolides E and F are nontoxic metabolites formed in shellfish (Hu *et al.*, 1996; Falk *et al.*, 2001; Aasen *et al.*, 2005) (Figure 69.15). Spirolides, produced by the dinoflagellate *Alexandrium ostenfeldii*, are accumulated in filter-feeding bivalves, such as mussels, oysters, and clams. They have been detected in the United States, Canada, Chile, Norway, Denmark, Scotland, Spain, France, and Italy (Cembella *et al.*, 2001; Munday, 2008c; Álvarez *et al.*, 2010).

Gymnodimines (Figure 69.16), containing a spiro center and an imine function, structurally related to spirolides, are produced by dinoflagellates of the *Gymnodinium* (*Karenia*) genus and accumulated in bivalves, such as oyster species (*C. gigas* and *Tiostrea chilensis*), Greenshell mussels (*Perna canaliculus*), blue mussels (*M. edulis*), and scallops (*Pecten novaezelandiae*), and also in tissues outside the digestive gland. These toxins have been detected in New Zealand and other countries, such as Tunisia and Canada (Munday, 2008c).

Pinnatoxins (Figure 69.17) are compounds consisting of a 20-membered ring with 5,6-bicyclo, 6,7-azaspiro, and 6,5,6-triketal moieties in their structure. They are



**FIGURE 69.16** Chemical structure of gymnodimine (from Quilliam, 2003).



**FIGURE 69.17** Chemical structure of pinnatoxin A (from Quilliam, 2003).

accumulated in shellfish of the genus *Pinna*, a common seafood in Japan and China. In these countries, consumption of *Pinna* caused human poisonings initially attributed to pinnatoxins (Kuramoto *et al.*, 2004) but that were later shown to be due to *Vibrio* species contamination (FAO/IOC/WHO, 2005). Pinnatoxins are structurally similar to the pteriatoxins, differing in that the cyclohexenyl side chain of the latter compounds ends in a cysteine. It has been suggested that pteriatoxins are biotransformed from pinnatoxins in shellfish (EFSA, 2010).

Other cyclic imines of algal origin are the cyclic polyether prorocentrolide (Figure 69.18) and its analog prorocentrolide B produced by dinoflagellates of the genus *Prorocentrum* (Munday, 2008c).

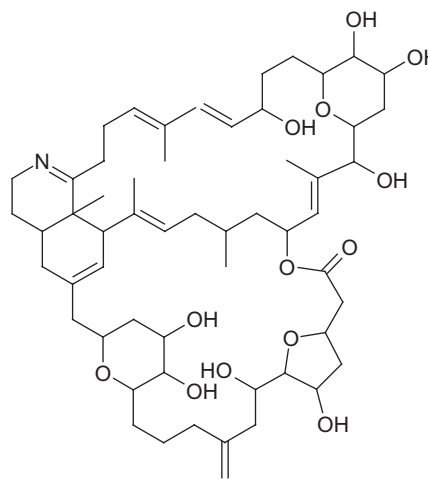
### Pharmacokinetics/toxicokinetics

No data on absorption, metabolism, distribution, and elimination of these cyclic imines are available.

### Toxicity

#### Human toxicity

No human poisoning syndrome due to cyclic imines has been documented. Due to the lack of adequate



**FIGURE 69.18** Chemical structure of prorocentrolide (from Torigoe *et al.*, 1988).

quantitative data on oral toxicity for cyclic imines, no ARfD has been established by the European Food Safety Authority Panel on Contaminants in the Food Chain (EFSA, 2010).

### Experimental toxicity

#### Single administration

After i.p. injection in mice, spirolides caused death within minutes, preceded by neurotoxic symptoms such as piloerection, abdominal spasms, hyperextension of the back, and arching of the tail (Gill *et al.*, 2003; Ciminiello and Fattorusso, 2004). By i.p. injection, spirolides B and D are of similar toxic potential, whereas spirolides E and F, in which the cyclic imine moiety is destroyed, are much less toxic. In mice, the LD<sub>50</sub> of a mixture of spirolides was calculated to be approximately 40 µg/kg after i.p. injection, with the lethality being approximately 25 times higher than that by the oral route (LD<sub>50</sub> = 1 mg/kg). The oral LD<sub>50</sub> values for pure desmethyl spirolide C, desmethyl spirolide C, and 20-methyl spirolide G were in the range of 157–176 µg/kg (Cembella *et al.*, 2002; Munday, 2008c).

For i.p. injection in mice, an LD<sub>50</sub> of 96 µg/kg was determined for gymnodimine, which induced paralysis and respiratory distress (Munday *et al.*, 2004). After administration in mice by gavage, an LD<sub>50</sub> of 755 µg/kg was calculated, but no toxicity was seen if the toxin was administered with finely ground food suspension at a level corresponding to a dose of approximately 7500 µg/kg (Munday *et al.*, 2004).

By i.p. injection in mice, the LD<sub>50</sub> of pinnatoxins E (45 µg/kg), F (16 µg/kg), and G (50 µg/kg), recently isolated from Japanese shellfish, was determined. Before death, mice were hypoactive and showed abdominal breathing, extension of the hind legs, low respiratory

rate, and exophthalmia (Selwood *et al.*, 2010). Data on acute i.p. toxicity of pteriatoxins in mice consist of an LD<sub>50</sub> of pteriatxin A (100 µg/kg), which was more toxic than a mixture (1:1) of pteriatoxins B and C (LD<sub>50</sub> = 8 µg/kg) (Munday, 2008c).

The “lethality” of prorocentrolide by i.p. injection in mice was reported to be 400 µg/kg, with death occurring within minutes (Torigoe *et al.*, 1988).

## Mechanism of action

The mechanism of action of the cyclic imines is not completely known. Biological studies on spirolides suggest hippocampus and brain stem as their main toxicity targets, with the toxins acting as muscarinic acetylcholine receptor antagonists and weak L-type transmembrane calcium channel activators (Gill *et al.*, 2003; Sleno *et al.*, 2004; Wandscheer *et al.*, 2010). The active pharmacophore of these compounds seems to be the cyclic imine moiety because spirolides E and F, lacking this structure, are inactive (Luckas *et al.*, 2005). Gymnodimine seems to block muscular and neuronal nicotinic acetylcholine receptors (Munday *et al.*, 2004; Kharrat *et al.*, 2008), and also pinnatoxins probably target these receptors at the neuromuscular junction (EFSA, 2010; Araoz *et al.*, 2011).

## Concluding remarks

Although cyclic imines are lethal in mice when injected i.p. and contaminate shellfish in several areas of the world, no cases of adverse effects in humans have been reported, and thus no maximum tolerated level for these toxins has been established (EFSA, 2010). The MBA has traditionally been used to detect cyclic imines in shellfish, and due to their high i.p. toxicity, these compounds complicate use of the MBA for lipophilic toxins. Other methods to quantify these toxins, such as LC-MS, have been set up to detect individual cyclic imines, but none of the current methods have been formally validated by interlaboratory studies (EFSA, 2010).

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# Botulinum neurotoxins

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## INTRODUCTION

Botulism, or “sausage poisoning,” was reportedly first recognized in Germany in approximately the late 1700s. However, it was not until the 1820s that Justinus Kerner systematically studied and described the fatal paralytic disease (aka, “Kerner’s disease”) associated with the ingestion of spoiled sausage. Kerner recognized that a poisonous substance isolated from the spoiled sausage was responsible for the clinical signs associated with botulism; however, he was unable to identify the origin of the deadly poison. With the subsequent introduction of anaerobic microbiological techniques in the late 1800s, the source of the poison was finally determined. In 1897, van Ermengem was able to identify the offending etiological agent as a bacterium, now known as *Clostridium botulinum*, in spoiled ham. The deadly poison produced by the bacteria is now known as botulinum neurotoxin (BoNT), the most potent biological toxin ever encountered, with lethal doses as low as 0.03 ng/kg body weight depending on both toxin type and animal species (Gill, 1982). This chapter describes the toxicology of BoNT in relation to various animal species.

## BACKGROUND

BoNT is produced under anaerobic conditions primarily by *C. botulinum*, a rod-shaped, spore-forming (subterminal) bacterium; however, other clostridial

species, such as *C. barati* and *C. butyricum*, are also capable of producing the neurotoxin (Hatheway, 1989). Although often referred to collectively as a single toxin, there are actually seven immunologically distinct serotypes of BoNT, and they are designated alphabetically, A–G (Simpson, 1981). Serotypes A, B, C1, and D have been associated with outbreaks of botulism in domestic animals, livestock, poultry, and wildlife, whereas serotypes A, B, E, and, rarely, F are known to cause disease in humans. When isolated from the bacterium, native BoNTs are found in complex with hemagglutinins and other nonhemagglutinin, nontoxic proteins. These accessory proteins are thought to protect the toxin from harsh environmental conditions such as those found in the gut following ingestion of toxin-contaminated food products. The molecular masses of these toxin complexes range between 300 and 500 kDa, depending on the toxin serotype. Although serologically distinct, the seven toxin serotypes share both structural and functional similarities. When not complexed with other proteins, the active neurotoxin has a molecular mass of 150 kDa and exists as a polypeptide di-chain molecule. The di-chain consists of a heavy chain linked by a single disulfide bond to a light chain. The 100-kDa heavy chain is responsible for membrane targeting and cellular uptake, whereas the 50-kDa light chain mediates its intracellular action. The neurotoxin molecule can be further divided both structurally and functionally into three domains. The heavy chain contains both a binding domain and a translocation domain, whereas the smaller light chain contains a catalytically active domain.

## MECHANISM OF ACTION

BoNTs are potent zinc-dependent metalloproteases that exquisitely target acetylcholine (ACh)-containing nerve terminals (Schiavo *et al.*, 2000). Because BoNTs are too large to cross the intact blood–brain barrier, their selective action on cholinergic terminals is generally limited to peripheral cholinergic systems. The primary target site is the neuromuscular junction (NMJ), where they act within the motor nerve terminal to prevent the release of ACh, the primary neurotransmitter at this major synapse. Intoxication by BoNT has been described as a multistage process (Simpson, 1981, 1989; Montecucco, 1986). In the first stage of intoxication, the toxins must bind selectively, via their heavy chains, to protein receptors (and gangliosides) located on the plasma membrane of the motor nerve terminal. The binding of the toxins to the nerve terminal membrane initiates the second stage of the intoxication process, which is characterized by receptor-mediated uptake of the toxins into endosomes (e.g., endocytosis). The third stage of intoxication occurs within the endosome, where the disulfide linkage between the toxin heavy and light chains is reduced, and a subsequent drop in endosomal pH promotes a conformational change in the toxin molecule, allowing the light chain to escape across the endosomal membrane into the cytosol, possibly mediated by the translocation domain of the toxin (e.g., translocation). Following this translocation to the cytosol, the toxin light chain is free to act upon its intracellular target during the final stage of intoxication. During this last stage of the process, the neurotoxins enzymatically cleave one of three specific proteins found within the presynaptic terminal. These three proteins – SNAP-25, synaptobrevin, and syntaxin – known collectively as SNARE (soluble *n*-ethylmaleimide sensitive factor attachment protein receptor) proteins, are necessary for neurotransmitter release. Synaptobrevin, an integral membrane protein, is found on the synaptic vesicle, whereas SNAP-25, a membrane associated protein, and syntaxin, another integral membrane protein, are both localized to the presynaptic terminal membrane. In normal circumstances, these SNARE proteins interact to form a four-helical “fusion” or “SNARE” complex that brings the ACh-containing synaptic vesicles into close apposition with the terminal membrane. Fusion of the vesicular and terminal membranes and the subsequent release of ACh are triggered by fast  $\text{Ca}^{2+}$  signaling. Proteolysis of any one of the SNARE proteins by BoNT either destabilizes or prevents the formation of functional SNARE complexes, inhibiting vesicular fusion and neurotransmitter release. The catalytic active sites of the different BoNT serotypes vary slightly, giving each serotype both substrate and cleavage site specificity. Toxin serotypes A, C, and E cleave SNAP-25; serotypes B, D, F, and G cleave synaptobrevin; and serotype C cleaves syntaxin.

## CLINICAL BOTULISM

Clinically, botulism is recognized as a lower motor neuron disease resulting in progressive flaccid paralysis. Although deficits in somatic neuromuscular transmission are the most prominent effects, motor deficits in cranial nerve function, as well as the autonomic nervous system, have also been reported. With the exception of impaired vision (most likely related to disruption in autonomic function), neither altered sensation nor mentation has been specifically reported in botulism. In animals, paresis begins in the hind limbs and progresses cranially, often resulting in quadriplegia and recumbency. As in humans, death may result from respiratory muscle paralysis. The onset of clinical signs may be within hours or days of exposure, and it is dependent on exposure conditions (dose and duration), as well as individual sensitivity. Susceptibility to the different serotypes varies across species. Botulism in large animals (herbivores) is commonly, but not exclusively, due to serotype B, whereas botulism in small animals (carnivores) and avian species is most commonly due to serotype C1. With the exception of serotype C1, the different toxin serotypes produce similar clinical disease. Serotype C1 toxin-producing strains of *C. botulinum* bacteria may produce multiple exotoxins, including the C1 neurotoxin, and the C2 and C3 cytotoxins. The concurrent presence of these cytotoxins with C1 neurotoxin may account for the different clinical scenarios that have been reported in botulism associated with serotype C1 intoxication, including dysautonomias in horses and cats.

Botulism is most often acquired from the ingestion of preformed toxin in either spoiled vegetation or carrion-contaminated foodstuffs, although this may vary among species. In addition, two other forms of botulism are well recognized in veterinary medicine: wound contamination with *C. botulinum* and gastrointestinal (GI) colonization by *C. botulinum* (toxico-infection). The mainstay of therapy for all animal species with botulism is supportive care, although the specifications of this therapy may differ slightly between species. Antitoxin (equine origin) may be administered in certain instances; however, because the antitoxin is only protective against toxin in the general circulation, it must be given early in the disease course to be effective. Once the patient displays symptoms, antitoxin administration may be ineffective. Furthermore, antitoxin administration can cause adverse effects. Antibiotic administration may be indicated to reduce the risk of secondary infections; however, aminoglycosides, tetracycline, and procaine penicillin should be avoided because these drugs potentiate neuromuscular weakness (L’Hommedieu *et al.*, 1979). *Clostridium botulinum* is not sensitive to metronidazole, an antimicrobial used to treat anaerobic infections. Furthermore, metronidazole has been associated



with an increased risk of botulism in laboratory animals and workers exposed to the bacterium, possibly due to alterations in GI flora that permit clostridial growth (Wang and Sugiyama, 1984; Whitlock, 2002).

## LABORATORY DIAGNOSIS

The diagnostic gold standard for botulism is the mouse bioassay (MBA). Although the MBA is still the most widely utilized test for botulism, alternative diagnostic avenues are being developed in an effort to minimize the use of animals in toxicity testing. For example, enzyme-linked immunoabsorption (ELISA) techniques have been used to identify BoNT in both avian and bovine specimens. For an in-depth discussion on this topic, the reader is referred to the review by Lindström and Korkeala (2006).

## SPECIES-SPECIFIC DISEASE

### Equine botulism

Horses are thought to be among the most susceptible of species to intoxication by BoNT. The serotype most commonly reported to cause equine botulism in North America (>80% of equine cases) is serotype B; however, serotypes A and C1 have also been associated with clinical disease in the horse (Whitlock *et al.*, 1989; Johnson *et al.*, 2010). Serotype B-producing strains of *C. botulinum* are found ubiquitously in the soils of the northeastern and central United States, particularly in regions extending from Kentucky to the mid-Atlantic states. Conversely, serotype A-producing strains are more prominent in the western states (California, Utah, Idaho, and Oregon) and have also been reported in Ohio. Although intoxication with BoNT/C1 occurs with less frequency, cases have been reported in California, Florida, the New England states, and Canada.

Horses acquire botulism in one of three ways: (1) from the ingestion of preformed toxin in contaminated foodstuffs, (2) from the contamination of wounds with *C. botulinum*, and (3) from the colonization of the intestinal tract with *C. botulinum* bacteria (toxico-infection). The ingestion of preformed toxin, in either spoiled vegetation or carrion-contaminated foodstuffs, is the most common scenario for equine botulism. Contaminated feed sources such as alfalfa cubes, alfalfa hay, baled hay, wheat, oats, potatoes, bale silage, rye silage, grass clippings, oat chaff, and brewer's grains have all been purported sources of botulism in large animals (Whitlock and Buckley, 1997).

Serotype B is more commonly associated with contamination of spoiled foodstuffs or moldy hay. Interestingly, the feeding of silage or hay stored in large plastic bags has been implicated as an increased risk factor for botulism. The damp, alkaline conditions of spoiled vegetation provide an optimal environment for clostridial growth, sporulation, and toxin production. For these reasons, it is recommended that silage with a pH greater than 4.5 should not be fed to horses (Whitlock and Buckley, 1997; Galey, 2001).

Cases of equine botulism associated with the ingestion of carrion-laden foodstuffs are more often the result of intoxication with BoNT serotype C1 (Galey, 2001). A herd outbreak of BoNT/C1 intoxication in California was determined to be the result of the ingestion of preformed toxin in alfalfa cubes contaminated with rodent carcasses (Kinde *et al.*, 1991). Interestingly, another report of an outbreak of BoNT/C1 intoxication in horses revealed that birds may act as vectors, transporting toxin or bacterial spores from a rotting carcass to nearby horse farms (Schoenbaum *et al.*, 2000).

Wound botulism occurs from the contamination of a wound with the *Clostridium* bacteria. The anaerobic environment of the wound provides optimal conditions for *C. botulinum* growth and toxin production. The neurotoxin (most commonly serotype B) is produced within the wound and enters the peripheral circulation, where it is distributed to the toxin's site of action, the NMJ. Distal limb wounds, castration sites, umbilical hernias, and injection site abscesses have all been associated with wound botulism.

"Shaker foal syndrome" is a form of toxico-infectious botulism that occurs in foals usually between 2 and 5 weeks of age (Rooney and Prickett, 1967; Vaala, 1991; Whitlock, 2002). The infection occurs most commonly in fast-growing foals on high planes of nutrition. GI ulcerations and liver abscesses have been documented post-mortem in foals that succumbed to botulism (Swerczek, 1980). Thus, exposure to stress, high nutrient diets, or corticosteroid use may all play a role in the foal's susceptibility to toxico-infection. Such underlying conditions may lead to gastric ulcers, which then serve as a nidus for *C. botulinum* colonization. Furthermore, as in human neonatal toxico-infection, the immature GI tract of foals may be more permissible to overgrowth by *C. botulinum*. Shaker foal syndrome is commonly associated with the production of toxin serotype B; however, a case of serotype C1 intoxication was documented in a foal in Florida (MacKay and Berkhoff, 1982). Serotype C1-producing clostridial strains have been identified in Florida soils, and in this particular case, it was concluded that the foal developed a toxico-infection after ingesting bacterial spores from the soil. In addition, this foal was also diagnosed with sand colitis; an irritated GI mucosa likely increased this foal's susceptibility to toxico-infection.

Toxico-infection with BoNT serotype C1 has also been implicated as a potential causative agent of equine dysautonomia, also known as equine grass sickness (EGS). EGS is frequently diagnosed in Great Britain, and its occurrence correlates with the grazing season. EGS presents as an acute or subacute illness resulting in death (or euthanasia) within days of onset; however, classic neuromuscular symptoms of botulism are not observed in EGS. Rather, the primary symptoms associated with this syndrome include dysphagia, ileus, and weight loss. If the horse survives the subacute phase, a chronic form may persist. In addition, histological examination revealed neuronal degeneration in autonomic ganglia, the myenteric plexus, and the submucosal plexus (McGorum *et al.*, 2003). Such histological findings are not reported with classic botulism. Increased BoNT/C1 has been detected in ileal and fecal contents of horses diagnosed with EGS; however, in this same study, BoNT/C1 was also isolated from a small number of control animals (Hunter *et al.*, 1999). Conversely, Garrett *et al.* (2002) demonstrated an increased number of bacterial colonies with a prominent number of clostridial species in the GI tract of horses with EGS compared with healthy control horses. However, the possibility that the increased colonization of the gut by clostridia was secondary to the ileus produced in EGS cannot be ruled out. McGorum *et al.* (2003) documented the occurrence of clinical and pathological signs associated with both EGS and toxico-infectious botulism in the same foal. One theory proposes that in addition to the blockade of ACh release at cholinergic nerve terminals, the C1 neurotoxin also causes nerve cell degeneration. However, note that serotype C1-producing clostridial strains may also produce the ADP-ribosylating cytotoxic toxins, C2 and C3. Interestingly, the C2 toxin has been associated with an increase in vascular permeability leading to the development of edema, congestion, and hemorrhage in *in vivo* models. It is plausible that these exotoxins may play a role in producing the clinical symptoms of EGS that deviate from those of classic botulism (Cottrell *et al.*, 1999; Hunter *et al.*, 1999; Garrett *et al.*, 2002). To date, however, the exact cause of EGS and the potential role of a *C. botulinum* toxico-infection in its manifestation remain elusive.

### **Clinical signs, diagnosis, and treatment**

The onset of clinical signs associated with equine botulism is variable and can occur anywhere between 12h and 10 days post-exposure. The clinical presentation of poisoned horses may be gradual, acute, or peracute depending on exposure dose and duration, as well as on individual sensitivity to the toxin. Adult horses that ingest low doses of toxin may show only mild dysphagia and recover with minimal treatment, whereas ingestion of large doses is associated with peracute illness and

a grave prognosis. In peracute illness, muscle paralysis progresses rapidly and the animal is recumbent within 8–12h; ultimately, paralysis of the respiratory muscles results in death.

With the exception of serotype C1 and EGS, clinical symptoms of equine botulism vary little between the different botulinum toxin serotypes (Whitlock, 1996; Whitlock and Buckley, 1997; Johnson *et al.*, 2010). Myasthenia and dysphagia are often the first symptoms observed. Astute horse owners may first notice mild signs such as depression, exercise intolerance, and difficulty with grain consumption. Ataxia, gait stiffness, and muscle tremors (particularly in the triceps muscles) may be noted early in the course of disease. In addition, mydriasis, ptosis, and decreased pupillary light responses, as well as decreased palpebral reflexes, are characteristic of early botulism. As the disease progresses, pupillary light responses diminish further. Periods of exercise may worsen paresis due to the reduction of ACh release at the NMJ. As dysphagia and pharyngeal weakness progress, the swallowing of food becomes more difficult and secondary aspiration pneumonia may ensue. Horses may also have difficulty drinking because they tend to stand with their muzzles submerged in water troughs without swallowing. In horses infected experimentally with serotype C, there was a more pronounced mydriasis and an inability to lift the head; facial edema and inspiratory stridor resulted from low head carriage.

Initially, vital signs such as heart rate, respiration rate, body temperature, and capillary refill time are within normal limits. However, decreased borborygmi, ileus, colic, and constipation develop as botulism progresses. Diarrhea is often associated with serotype C, possibly in association with C2 toxin. Urine retention with resultant bladder distention often occurs, thereby increasing the risk of urinary tract infection. As the disease progresses, horses spend more time in sternal recumbency and ultimately become laterally recumbent. Heart and respiratory rates may increase as recumbent horses struggle to stand. In late stages, dyspnea and other signs of respiratory distress may be observed. With serotype C intoxication, an exaggerated expiration and “prolonged abdominal lift” may be noted. In the final stages of botulism, horses are laterally recumbent, demonstrate significant respiratory difficulty, and develop anoxia. As the anoxia progresses, horses may exhibit agonal paddling. At this point, the patient either dies due to respiratory failure or is euthanized.

In the foal, botulism most commonly occurs between 2 and 5 weeks of age. The first clinical signs usually observed are increased periods of recumbency and muscle tremors. Soon after the foal rises, muscle tremors are evident, and after brief periods of standing, the foal collapses from weakness. Recumbent foals appear

to be bright and alert. Foals may dribble milk from their muzzles soon after nursing due to dysphagia and pharyngeal muscle paresis. Thus, aspiration pneumonia is a common sequela in the foal. Constipation and ileus are also frequently observed. Other symptoms are similar to those observed in the adult horse.

A tentative diagnosis of botulism can be made following a comprehensive neurological assessment. Typically, abnormalities in palpebral reflexes, pupillary light responses, tail tone, tongue tone, prehension of food, dysphagia, and gate are detected with botulism. Both the tongue stress test and the grain test are sensitive measures for botulism. Difficulty in prehension, slow consumption, and a characteristic grain/saliva mixture hanging from the lips are indicative of botulism. These examination findings may also be useful in the assessment of disease progression and treatment efficacy.

Abnormalities in routine diagnostic indicators (complete blood cell (CBC) count, blood chemistry, cerebral spinal fluid (CSF) analysis, and urinalysis) are not typically detected in early botulism, but they do usually accompany other neurological diseases. Therefore, normal laboratory values in light of neurological deficits support the diagnosis of botulism. Differential diagnoses for botulism include infectious diseases such as equine protozoal myeloencephalitis, equine herpes virus-1, eastern and western equine encephalitis, rabies, guttural pouch mycosis, and listeriosis; other toxicoses such as leukoencephalomalacia (moldy corn poisoning), ionophore poisoning (monensin, salinomycin, and narsin), yellow star thistle poisoning, yew poisoning, white snake root poisoning, and organochlorine poisoning; metabolic disorders such as equine motor neuron disease, azoturia, eclampsia, hypocalcemia, hyperkalemic periodic paralysis, and white muscle disease; and pharyngeal ulceration.

A tentative diagnosis of equine botulism may be confirmed by (1) MBA detection of formed toxin in horse sera, GI contents, viscera, or wounds; (2) detection of *C. botulinum* spores or toxin in suspect foodstuffs in association with clinical signs; and (3) ELISA detection of serum anti-toxin antibodies in unvaccinated horses with clinical signs. However, a definitive diagnosis of botulism is often difficult to achieve in the horse. There are no gross or pathognomonic histological lesions associated with botulism, and serum toxin levels in the horse are often too low to be detected by the MBA. Because the horse is more sensitive than the mouse to BoNT, the MBA is most valuable in early, peracute equine botulism, when higher concentrations of toxin may be present in the bloodstream. In addition to serum, GI contents and liver samples can also be submitted for the MBA, although greater diagnostic success may be achieved through detection of BoNT in the foodstuff rather than within the patient (Galey, 2001). Fecal or tissue culture enrichment

can be used to enhance bacterial spore numbers and toxin levels for greater detection. However, because spores may be present in the feces of healthy horses, direct detection of BoNT within the animal is a more reliable finding. Following a positive result from the MBA, the serotype can be identified using the mouse neutralization test.

Once botulism is suspected, the patient should be confined to the stall to prevent exertion. Polyvalent antiserum (antitoxin) should be given as soon as possible; the recommended dose for an adult horse is 70,000 IU (500 mL), and that for the foal is 30,000 IU (200 mL). One dose usually provides passive immunity for approximately 60 days (Sprayberry and Carlson, 1997; Whitlock and Buckley, 1997). The use of parasympathomimetics should be avoided because these agents deplete ACh stores and exacerbate paresis/paralysis. Antibiotic therapy is indicated in cases of wound botulism or secondary infections; however, as previously stated, aminoglycosides, tetracycline, procaine penicillin, and metronidazole are contraindicated. Aminoglycosides block neurotransmission at the NMJ and will exacerbate muscle weakness and paralysis (Barsanti, 1990). Although gram-positive anaerobes are sensitive to penicillin and metronidazole, administration of these drugs is controversial. These antimicrobials may cause more bacterial lysis, thus increasing the release of toxin (in the case of a toxico-infection), or they may promote *C. botulinum* colonization by altering the normal intestinal flora. Drugs such as the aminopyridines and guanidines should also be avoided because they will further deplete ACh stores (Critchley, 1991).

Second only to antitoxin administration, supportive care is the mainstay of therapy for botulism. H<sub>2</sub> blockers and proton pump inhibitors may be indicated, especially for foals. Topical ophthalmic ointments should be used to prevent corneal abrasions and ulceration. Adult horses may need to be sedated with xylazine or diazepam to reduce anxiety and exertion. Patients should be muzzled between feedings to prevent aspiration pneumonia. Nutritional support should be provided to dysphagic patients. Alfalfa slurries with adequate amounts of water may be administered through a nasogastric tube to adult horses. Foals should receive milk replacer through a nasogastric tube or parenteral nutrition if ileus is present. Patients should be maintained in sternal recumbency to prevent aspiration pneumonia and checked periodically for gastric reflux because ileus may lead to the accumulation of ingesta/fluid in the stomach. If gastric reflux is not present, some authors recommend that mineral oil be administered via a nasogastric tube to alleviate ileus and constipation; however, this should be done under close supervision due to the increased risk of aspiration in these patients. Recumbent patients should be turned frequently or suspended periodically by full-body slings to prevent decubital ulcer formation, myopathies, and other

complications of prolonged recumbency. Recumbent stallions and geldings should be catheterized twice daily to empty the bladder and prevent pressure necrosis or cystitis (Whitlock and Buckley, 1997).

A tracheostomy should be performed in cases of botulism in which horses show signs of upper airway obstruction as a result of paralysis of the nares or larynx. In more complicated cases, patients may require intravenous (i.v.) fluids to correct respiratory acidosis resulting from decreased ventilation. For foals in particular, arterial blood gases should be monitored frequently to determine the need for artificial ventilation. Intranasal oxygen insufflation and mechanical ventilation can be instituted in foals with poor arterial blood gas values and/or metabolic acidosis (Wilkins and Palmer, 2003). Unfortunately, mechanical ventilation is not practical in the adult horse (Mitten *et al.*, 1994).

The overall prognosis is favorable for horses that are exposed to low doses of the toxin, exhibit a slow disease progression (3–7 days), or display mild symptoms. Likewise, a grave prognosis is given to patients exposed to high doses of toxin, that manifest a rapid onset of clinical disease, or that become recumbent within 8–12 h. Patients responsive to antitoxin therapy should be able to eat within 7–10 days post-treatment and regain full strength within a month. Recumbent foals are often able to stand within 7–10 days post-antitoxin administration. Although the prognosis for recumbent adult horses is poor, if the patient does not become distressed or show severe respiratory compromise, recovery may be achieved with extensive supportive care. The most common complications associated with botulism are decubital ulcers and aspiration pneumonia; these problems can be resolved with supportive care and antimicrobial therapy.

### Prevention

Following recommended vaccination protocols, along with sound husbandry methods, reduces the occurrence of equine botulism. Forages should be examined for carrion, and pastures should be cleared of decaying vegetation and rotting animal carcasses. Appropriate wound management is also an important preventative measure. To date, only serotype B toxoid vaccine is marketed for horses in the United States. The American Association for Equine Practitioners recommends vaccination only for horses in endemic areas. Adult horses in endemic areas should be vaccinated annually. Mares should be boosted 4–6 weeks prior to parturition to achieve adequate antitoxin immunoglobulin (Ig) levels in colostrum. Foals born to vaccinated mares should receive a series of three vaccinations, each 1 month apart, starting at 2 or 3 months of age. Foals born to unvaccinated mares should be vaccinated at 2, 4, and 8 weeks of age (Whitlock and Buckley, 1997; Galey, 2001).

### Avian botulism

Avian botulism, otherwise known as “limberneck” or “western bird disease,” has been a significant problem worldwide in both domestic and wild fowl. The occurrence of avian botulism has been globally widespread, having been documented in as many as 17 countries and on every continent except Antarctica (Jensen and Price, 1987). The majority of the natural outbreaks of avian botulism have occurred in fowl (Lamana, 1987). Carnivorous, omnivorous, carrion scavengers, and insectivorous birds, as well as aquatic bottom-feeding birds, are all susceptible to botulism. In 1984, 117 avian species were determined to have been affected by the disease. Specifically, botulism has been reported in chickens, ducks, turkeys, pheasants, and ostriches. Although broiler outbreaks are not uncommon, botulism is a more significant problem for waterfowl, resulting in millions of deaths worldwide. Avian species are sensitive to serotypes A, B, C1, and E, although serotype C1 is most commonly associated with outbreaks. Outbreaks of serotype C1 intoxication have been reported worldwide, whereas outbreaks of serotype A botulism have only been reported in western regions of North and South America; serotype B in the eastern United States, England, Europe, and China; and serotype E in the Great Lakes and North Sea. Interestingly, serotype A was found to be more toxic than serotype C1 when administered i.v. to chickens; however, when given orally, serotype C1 demonstrated greater toxicity.

The etiology of botulism among wild avian species and waterfowl differs from that observed in other animals. The process is a complex cycle involving environmental contamination, toxico-infection, bird die-offs, bacterial proliferation in bird carcasses, and invertebrate vectors. *Clostridium botulinum* often colonizes the intestinal tract and cecum of clinically normal birds, increasing the potential for toxico-infection in avian species (Dohms, 2003). Because these birds are already seeded with the bacteria, upon death, avian carcasses provide an excellent substrate for *C. botulinum* growth. The proliferating bacteria spread from the GI tract to other tissues, the carcass becomes flyblown, and toxin accumulates in the fly larvae. Invertebrates concentrate the bacterium or toxin after feeding on contaminated carcasses; however, due to their neurophysiological differences, BoNT does not affect insects and aquatic invertebrates. Subsequently, birds ingest these animals and accumulate lethal amounts of BoNT. One gram of fly larvae may contain  $1.8 \times 10^5$  mouse LD<sub>50</sub>s, and ingestion of as little as eight fly larvae was sufficient to kill a pheasant. Bird and invertebrate die-offs perpetuate botulism outbreaks by increasing the levels of *C. botulinum* in soils, lakes, rivers, and estuaries. Environmental factors such as shallow alkaline waters, warm seasons/summer



months, and flooding of mudflats or dried-out lakes may promote invertebrate die-offs, further enhancing environmental levels of *C. botulinum*. As *C. botulinum* levels increase in the environment, the intestinal tracts of wild birds and waterfowl become seeded with the bacteria, and any cause of bird deaths can trigger an outbreak of botulism.

Contaminated feed, water, litter, carcasses, and insects may be associated with botulism in broilers. Often, the source of BoNT cannot be identified, and toxico-infection has been hypothesized to be the perpetuating factor. *Clostridium botulinum* has been isolated from the intestinal tract and cecum of healthy birds; furthermore, the chicken body temperature (41°C) and cecal pH (7.4) are optimum for *C. botulinum* growth (Miyazaki and Sakaguchi, 1978; Trampel *et al.*, 2005). Most broiler outbreaks have occurred in chickens between 2 and 3 weeks of age; however, an outbreak in postcaponized chickens was documented in birds as old as 14 weeks. Coprophagy has also been implicated as a causative factor in poultry outbreaks because both BoNT/C1 and *C. botulinum* are secreted in cecal droppings. Broiler outbreaks are also more likely to occur in hot weather.

Morbidity and mortality of avian botulism increase with the dose of BoNT ingested. The onset of clinical symptoms may be from a few hours to 2 days post-exposure. The mortality rate in broilers has been reported to be as high as 27%, whereas thousands to millions of birds may have been lost as a result of outbreaks in waterfowl. In fact, it had been suggested that botulism may have been the limiting factor of waterfowl population growth in predisposed areas of the United States (Jensen and Price, 1987).

### *Clinical signs, diagnosis, and treatment*

As in other species, avian botulism is characterized by lower motor neuron deficits resulting in flaccid muscle paralysis. Paresis begins in the legs and progresses cranially to involve the wings, neck, and eyelids. Mildly affected birds may appear ataxic, reluctant to move, have a ruffled coat, and have easily epilated feathers. The wings may droop and the neck may become flaccid, hence the name “limberneck.” Diarrhea is often noted in broilers. As the disease progresses, birds become recumbent. Neck muscles become paralyzed, and birds eventually lie down with necks extended out, resting on the ground. Birds may appear comatose due to eyelid paralysis. Dyspnea may develop as paralysis progresses. Birds usually die from respiratory failure and dehydration. Broilers may succumb to hyperthermia as sick birds are smothered by others and the respiratory mucosal cooling mechanism is compromised.

The diagnosis of avian botulism is based on clinical signs, a lack of specific pathological changes, and

the isolation of toxin from serum/tissues of clinically ill birds. Although no pathognomonic changes have been described, postmortem hepatic and renal congestion along with signs of dehydration may be found. The most definitive diagnosis of botulism is the isolation of BoNT from the sick bird. Ten milliliters of blood is the suggested minimum amount for the MBA; however, if necessary, equal aliquots of blood from individual sick birds may be pooled to accommodate volume requirements of the assay (Dohms, 1987). Following a positive result from the MBA, the serotype can be identified using the mouse neutralization test. Most outbreaks of avian botulism are due to BoNT/C1; therefore, antiserum for serotype C1 is usually tested first. One IU of antiserum/mouse typically neutralizes BoNT/C1 levels found in chickens suffering from botulism. BoNT/C1 and other serotypes can also be detected in bird serum using ELISA technology. For small sample volumes, the MBA appears to be more sensitive; however, for larger serum samples, the ELISA sensitivity may be comparable to the MBA. Isolation of BoNT or *C. botulinum* from the bird intestines, cecum, or other tissues may aid in a diagnosis; however, these tests are less valuable because the bacterium can be isolated from the intestinal tract of healthy birds. Furthermore, isolation of BoNT or the bacterium from carcass tissues is not definitive because *C. botulinum* may proliferate and spread from the intestinal tract to surrounding tissues of the carcass. The MBA can be performed on intestinal, cecal, and crop flushes, or samples can be assayed for toxin or bacterium after culture enrichment. Polymerase chain reaction methods have been used to identify genes encoding BoNT/C light chain in cecal contents. In order to identify the source of contamination, feed, water, litter, carcasses, and insects should be assayed for toxin or cultured to isolate the bacterium. Both ELISA and the passive hemagglutination test can be performed to identify serum antibodies to BoNT. However, the levels of toxin that produce illness are usually insufficient to stimulate an immune response in chickens and ducks. Interestingly, antibody titers to several BoNT serotypes have been identified in healthy carrion-eating birds such as vultures and crows (Ohishi *et al.*, 1979).

Differential diagnoses for avian botulism in poultry include transient brain paralysis, coccidiostat toxicity, pesticide or other chemical toxicity, New Castle disease, Marek's disease, avian encephalomyelitis, avian reovirus, and musculoskeletal problems. Fowl cholera and chemical toxicity, particularly lead poisoning, are the common differentials for botulism in waterfowl. However, eyelid paresis and the lack of postmortem lesions are supportive of botulism as the diagnosis.

When possible, clinically ill birds should be isolated and provided fresh water; once these measures are taken, birds often recover fully within a few days.

Waterfowl should be herded to uncontaminated shores, and carcasses should be removed daily in poultry operations. Antitoxin therapy may be administered for valuable birds or zoo animals, but it is impractical for most production operations or wildlife. Furthermore, antitoxin protection is transient, and birds may again become susceptible to BoNT. In broiler outbreaks, antimicrobial therapy may be instituted through watering systems or feed. Administration of bacterin (100 g/ton of feed) or streptomycin (500–1000 g/ton of feed or 1 g/L of water for 3 days) was shown to decrease mortality rates in chickens (Schettler, 1979; Sato, 1987). Penicillin may also be administered, but a mixed efficacy has been reported with this treatment. Periodic use of chlortetracycline was reported to reduce botulism outbreaks on one poultry farm. Additives such as sodium selenite (6 g/1000 L of water for 5 days) and vitamins A, D<sub>3</sub>, and E may also reduce mortality (Schettler, 1979). Conversely, elevated iron levels in water or feed may promote the intestinal proliferation of *C. botulinum*; therefore, citric acid, an iron chelator, may be added to water as a preventative (Pecelunas *et al.*, 1999). Furthermore, citric acid may lower the pH of the GI tract, inhibiting the growth of *C. botulinum* and promoting the growth of normal flora.

### Prevention

Immunization with the toxoid vaccine has been explored in broilers, pheasants, and ducks with mixed results (Shimizu and Kondo, 1978). Protection in broilers between 3 and 8 weeks of age was variable after vaccination at 1 and 14 days of age. Chickens are most susceptible to botulism between 2 and 8 weeks of age, and vaccinations to protect this group may be less efficacious due to interference from maternal antibody and immaturity of the immune system. Routine vaccination further increases production costs, and the toxoid may not provide adequate protection against the high doses of toxin obtained from maggot ingestion. Toxoid immunizations are also impractical for waterfowl. Therefore, preventative measures to minimize outbreaks of avian botulism should be aimed at flock and environmental management in both production birds and waterfowl.

In broiler outbreaks, the goals are to limit further exposure and eliminate *C. botulinum* or BoNT from the environment. Unaffected birds should be moved to uncontaminated houses. Carcasses should either be incinerated or buried in a deep hole. Rodents should be eliminated from broiler houses because rodent carcasses may harbor *C. botulinum*. Chicken houses associated with outbreaks should be emptied and cleaned. All litter should be removed. Houses should be washed with high-pressure steam and cleaned with a detergent agent. A surface-active solution should be sprayed on the

interior walls. The walls should then be disinfected with an organic iodine solution or an organic iodine and calcium hypochlorite solution. Twenty-four hours later, the interior walls should be sprayed with 10% formalin. Soil in contaminated areas may also be treated with calcium hypochlorite. Houses should also be sprayed with pesticides to limit flies. Iron levels in feed and water sources should be monitored.

Prevention of waterfowl outbreaks is best achieved by reducing the potential for environmental contamination associated with the proliferation of *C. botulinum* in the carcasses of dead vertebrate and invertebrate animals. Carcasses should be collected, and flocks should be herded away from shores associated with outbreaks. Pond management should maintain deep waters, steep banks, and smooth bottoms to prevent deaths of invertebrates and vertebrates. Routine flooding, which may lead to the death of terrestrial invertebrates, should be avoided in areas utilized by waterfowl. Water in wetland areas should be maintained as fresh as possible because oxygen depletion in shallow, stagnant waters leads to aquatic animal die-offs. Any factors that may increase deaths in susceptible wetlands, such as overhead power lines, should be removed or avoided.

The possibility for transmission of botulism from birds to their predators may exist. Coincidence of avian outbreaks with botulism in omnivorous animals has been documented. For instance, Weiss *et al.* (1982) reported botulism in a fox and a weasel in association with a waterfowl outbreak. In addition, there have been several reports of canine botulism in hunting breeds (Barsanti *et al.*, 1978; Richmond *et al.*, 1978; Jensen and Price, 1987). Farrow *et al.* (1983) reported the occurrence of botulism in three dogs after the consumption of a rotten duck carcass. Outbreaks of botulism (BoNT/C and D) in cattle and sheep have been associated with the feeding of contaminated poultry litter in silage (Egyed, 1987; McLoughlin *et al.*, 1988). No cases of human botulism resulting from the consumption or handling of contaminated birds have been reported, although both scenarios have likely occurred. The risk for the human acquisition of botulism from avian species appears to be limited. Although Smart *et al.* (1980) reported an outbreak of BoNT/C in nonhuman primates, humans do not appear to be susceptible to BoNT/C or D following oral exposure (Jensen and Price, 1987). Furthermore, proper cooking of poultry should denature any toxin protein and eliminate the possibility of transmission through consumption.

### Bovine botulism

Cattle are susceptible to BoNT serotypes B, C1, and D, and they acquire botulism most commonly from the

ingestion of preformed toxin in spoiled silage, carrion-laden silage, or silage contaminated with poultry litter. As with horses, toxico-infectious and wound botulism are also potential routes of intoxication in cattle. All three toxin serotypes (B, C1, and D) have been associated with clinical disease caused by the ingestion of spoiled or carrion-laden feedstuffs. Intoxication with BoNT/B is associated with the ingestion of poorly ensiled or spoiled silage, whereas BoNT/C1 is associated with the ingestion of carrion or poultry litter-laden feedstuffs. Although less frequent, BoNT/D has also been implicated with the ingestion of contaminated silage. Interestingly, intoxication with BoNT/D has also been associated with the ingestion of bones by phosphorus-deficient cattle or cattle with pica. Numerous studies have documented the association between outbreaks of bovine botulism and the ingestion of improperly ensiled silage or spoiled haylage contaminated with BoNT/B (Divers *et al.*, 1986; Yeruham *et al.*, 2003; Braun *et al.*, 2005). Wet hay or soil-contaminated hay, wrapped in plastic bags for storage, can provide the ideal moist anaerobic environment for *C. botulinum* growth. Contamination of a total mixed ration (TMR) with a cat carcass was determined to be the source of a BoNT/C1 outbreak in a herd of adult Holstein dairy cattle in California (Galey *et al.*, 2000). The practice of feeding ensiled poultry litter to cattle has also been associated with outbreaks of serotype C1 botulism, as documented in an Irish beef herd by McLoughlin *et al.* (1988). Although serotype D is less commonly associated with food-borne botulism, an outbreak occurred on a Canadian feedlot following the feeding of a TMR containing spoiled bakery waste (Heider *et al.*, 2001). A separate outbreak of BoNT/D in a Holstein dairy herd occurred in which the source was suspected, but not proven, to be contaminated haylage (Martin, 2003).

### Clinical signs, diagnosis, and treatment

Bovine botulism usually occurs in the context of a herd outbreak. The classical signs of bovine botulism are similar to those observed in horses; however, cattle exhibit a more gradual progression of clinical signs, improving the prognosis and probability of recovery in cattle (Whitlock *et al.*, 1989; Whitlock and Williams, 1999). Furthermore, at least one study has reported that ruminal microbes degrade BoNT, decreasing the absorption of active toxin in cattle compared to horses (Allison *et al.*, 1976). The clinical course ranges from 2 to 30 days, depending on exposure dose and duration and the administration of treatment. Early botulism may be confused with milk fever because generalized muscle weakness, increased ataxia, and muscle tremors may occur in both conditions. Cattle with botulism also exhibit depression, dysphagia, decreased tongue and

jaw tone, hypersalivation, dehydration, decreased tail tone, decreased pupillary light responses, and mydriasis. Rumen contractions decrease, and constipation may develop. Diarrhea and/or putrid-smelling feces may also be noted. Cattle with botulism tend to spend significant amounts of time in sternal recumbency. At terminal stages of botulism, cattle are laterally recumbent, exhibit abdominal breathing patterns, and finally succumb from respiratory failure. Vital signs are often normal in early stages of botulism; however, as the disease progresses, increased heart and respiratory rates may be noted, whereas body temperature may decrease.

A syndrome resembling equine dysautonomia has been described in German cattle, and a link to BoNT has been proposed. These cattle may present with a subclinical to chronic "visceral" disease. Nonspecific symptoms such as weight loss, decreased milk production, depression, alternating constipation and diarrhea, edema, laminitis, ataxia, retracted abdomen, emaciation, tachypnea, and unexpected death are associated with this syndrome (Böhnel *et al.*, 2001). In cattle exhibiting these symptoms, Böhnel and associates demonstrated the presence of both *C. botulinum* and BoNT in lower GI tract contents. Furthermore, neither BoNT nor *C. botulinum* was isolated from asymptomatic herds. This study hypothesized that small levels of *C. botulinum* colonized the lower intestinal tract and created a low-level, chronic exposure of BoNT. This low level of toxin may not reach the systemic circulation, and thus toxin may only disrupt nearby parasympathetic ganglionic innervation of the GI tract, altering intestinal function.

In cases of bovine botulism, clinical pathology may reveal signs of dehydration such as increases in packed cell volume and total plasma protein. Bicarbonate loss from excessive ptyalism may lead to a metabolic acidosis. Increases in muscle enzymes such as aspartate transaminase and creatinine kinase may be present due to muscle atrophy or trauma resulting from prolonged lateral recumbency. Electrolyte abnormalities and hyperglycemia may also be detected. One study documented indicators of renal failure in a herd poisoned by BoNT/B; increased  $\gamma$ -glutamyl transpeptidase, urea, creatinine, and phosphorus were also detected.

As with other species, there are no definitive gross pathological or pathognomonic histological signs of botulism. Aspiration pneumonia and pulmonary emphysema are the most frequent sequelae of botulism in cattle (Galey *et al.*, 2000; Heider *et al.*, 2001; Braun *et al.*, 2005). Other lesions, such as gastric ulcerations, thickened intestinal mucosa, hepatic lipidosis, suppurative rumenitis, and renal failure, have been documented in concurrence with botulism; however, these findings are not consistent in all cases of bovine botulism.

Botulism in cattle is usually a presumptive field diagnosis made on the basis of clinical signs and the ruling

out of other diseases. Differential diagnoses include hypocalcemia, hypomagnesemia, hypokalemia, hypophosphotemia, listeriosis, lead poisoning, polioencephalomalacia, ionophore toxicity, nutritional or plant toxin-induced myopathies, molds, organophosphate poisoning, and tick paralysis. Clinical diagnosis is usually made through the detection of neurological deficiencies in light of relatively unremarkable laboratory diagnostic findings. The neurological examination should assess cranial nerve responses, gait, posture, and attitude. Specifically, a tongue tone test, tongue stress test, and a jaw tone test should be performed. The tongue stress test is performed by placing a hand at the base of the cow's tongue and putting pressure on the tongue followed by an assessment of muscular tone. The tongue tone test is performed as in the horse. Cattle with botulism will exhibit weak tongue strength. The jaw test is performed by grasping the mandible near the symphysis and attempting to move the mandible laterally. This test assesses the strength of the masseter muscles. A "loose" jaw is suggestive of botulism.

A definitive diagnosis is made by identifying toxin in the patient's serum, ruminal fluid, or tissues. Identification of BoNT or *C. botulinum* in suspect feedstuffs previously consumed by clinically ill animals may further support a diagnosis. Isolation of BoNT from the rumen may prove difficult because the toxin is often diluted by rumen contents and/or degraded by ruminal microbes. Similar to botulism in other species, the MBA is the gold standard for a definitive diagnosis in cattle; however, as in horses, the MBA is often not sensitive enough to detect the low levels of toxin in the general circulation. The MBA may also be used to detect toxin in rumen contents, the liver and other organ tissues, milk, or feedstuffs. Due to the relatively slower progression of clinical signs in cattle, diagnostic samples are often obtained long after ingestion of toxin. Thus, the level of toxin in these samples may have fallen below the level of detection. Specimens may also be cultured to isolate *C. botulinum*. An ELISA test has been developed to detect BoNT/C and D in cattle; however, this test is considered less sensitive than the MBA. ELISA tests for the detection of antibodies to BoNT/C and D in cattle have also been developed.

Supportive care is the core of therapy for bovine botulism, and treatment should only be pursued in standing cattle. Affected cattle should be kept in confinement to minimize movement and exertion. Dehydration, electrolyte deficiencies, acid-base abnormalities, and glucose deficiencies should be managed with fluid therapy. Fluids can be administered orally (via an orogastric tube) or i.v. Mineral oil or sodium sulfate can be administered with care as cathartics to treat ileus; however, magnesium sulfate should be avoided because it may potentiate muscle weakness. Rumen transfaunation may also

be performed. Alfalfa gruels may be administered via an orogastric tube to maintain caloric intake. Equine origin polyvalent antiserum may be administered to cattle. However, antitoxin therapy may be less efficacious in cattle because most of the toxin will have been internalized into the neuron or degraded by the time the diagnosis is made. Antibiotics may be administered for secondary complications such as aspiration pneumonia; as in horses, those that produce muscle weakness should be avoided. Although toxoid vaccinations for serotypes B, C, and D are administered to cattle in other countries, there are no U.S. Food and Drug Administration-approved vaccinations for cattle in the United States.

### Public health

The Food Safety Act of the United States (1990) requires that meat or milk products be withheld from market for a minimum of 14 days after the onset of the last clinical case of botulism in an affected herd (Cobb *et al.*, 2002). However, the public health concern for transmission of BoNT through milk appears to be minimal. No cases of human botulism acquired from the consumption of meat or milk from botulism-affected cattle have been reported; furthermore, it does not appear that calves acquire botulism through nursing from affected cows. Only a single study to date has been able to detect BoNT in milk from a dairy cow affected with botulism (Böhnel *et al.*, 2005). In this study, BoNT/B was isolated in milk collected from one udder quarter that was simultaneously affected with mastitis. The toxin concentration in the milk was determined to be approximately  $10^4$  mouse LD<sub>50</sub>s. However, the milk did not test positive for *C. botulinum* bacteria. It is likely that the concurrent mastitis infection enhanced the passage of the rather large toxin protein (150 kDa) across the normally protective blood:milk barrier by altering its permeability. This is supported by a much earlier report from Moberg and Sugiyama (1980), who isolated BoNT in milk using an infected rat model. Other studies have not been able to detect BoNT in milk from affected cows using either ELISA or MBA techniques (Galey *et al.*, 2000; Cobb *et al.*, 2002; Moeller *et al.*, 2003). Regardless, the pasteurization process would likely denature any toxin protein that was able to pass into milk, reducing the risk to the consumer. Note, however, that the potential for BoNT contamination of milk may be more relevant in regions where unpasteurized milk is available for public consumption.

### Canine and feline botulism

Although carnivores are thought to be more resistant to the development of botulism, cases of canine botulism have been documented in the United States, Great Britain,



continental Europe, and Australia. Most reported cases of botulism in dogs result from the ingestion of BoNT/C1-contaminated carrion; however, a few cases of serotype D have been documented in Senegal (Barsanti, 1990, 2006). Barsanti *et al.* (1978) described an outbreak of type C1 botulism in a hunting colony of American foxhounds; however, the source of the toxin was not identified. Farrow *et al.* (1983) described type C1 botulism in three young Australian cattle dogs following the ingestion of rotting duck carcasses found in a local Sydney park. Canine botulism has also been associated with the ingestion of contaminated raw meat (Darke *et al.*, 1976).

Until recently, the only documented cases of feline botulism were experimentally induced; however, Elad *et al.* (2004) described a natural outbreak of botulism in eight cats that ingested parts of a pelican carcass contaminated with BoNT/C1. Interestingly, BoNT/C1 botulism has also been reported in lions (Critchley, 1991).

### Clinical signs, diagnosis, and treatment

The onset of canine botulism can occur within hours or as late as 6 days post-exposure. Severe cases are associated with an earlier onset of clinical signs. The course of the disease usually ranges from 12 to 24 days. In the clinical report of an outbreak of feline botulism, clinical symptoms were first noted 3 days post-ingestion of contaminated pelican muscle (Elad *et al.*, 2004). Although 50% of the exposed cats died, those that survived recovered significantly by 6 days post-intoxication.

Lower motor neuron dysfunction and, to a lesser extent, cranial nerve and autonomic nervous system deficits are observed in canine botulism. Paresis begins in the hind limbs and progresses cranially, ultimately resulting in flaccid muscle paralysis and quadriplegia. Interestingly, dogs with botulism maintain the ability to wag their tail. Tremors of the masseter and temporal muscles may be noticed. Muscle atrophy is variable throughout the course of the disease. Mydriasis, decreased pupillary light response, decreased palpebral reflexes, and decreased or weak vocalizations may occur. Hyperemic conjunctiva and decreased Schirmer tear tests may be noted. Heart rates and respiratory patterns are variable; however, as abdominal muscle tone diminishes, diaphragmatic breathing may be noted. Regurgitation, megaesophagus, urinary retention, and constipation are also observed. Secondary complications include aspiration pneumonia, bilateral keratoconjunctivitis sicca, and urinary tract infections. If paralysis progresses to the respiratory muscles, death may occur from respiratory failure; however, death may also result from progressive secondary pneumonia or urinary tract infections. If secondary complications do not arise, the prognosis for canine botulism is good. Recovery occurs in the reverse order from that of the onset of paralysis; cranial

nerve function and motor function of the neck and limbs return. In the one case study of natural botulism in cats, clinical signs were similar to those of dogs. Motor deficits and paresis were noted; however, cranial nerve reflexes were normal. Depression, anorexia, mild dehydration, tachycardia, and urinary retention were also noted.

As with EGS in the horse, there has also been speculation of an association between feline dysautonomia (Key-Gaskell disease) and BoNT/C. Clinical signs for feline dysautonomia include depression, anorexia, vomiting, regurgitation, mydriasis, constipation, and urinary retention; however, the somatic lower motor neuron paralysis characteristic of classical botulism is not observed. Histological evidence of neuronal degeneration in autonomic ganglia confirms the diagnosis of dysautonomia. Interestingly, BoNT/C was detected in feces, ileal contents, and foodstuffs of cats displaying symptoms of dysautonomia (Nunn *et al.*, 2004). Furthermore, affected cats had higher levels of anti-BoNT/C and *C. botulinum* surface antigen IgA in their feces compared to control cats. Additional studies are warranted to determine the potential role of BoNT/C in feline dysautonomia.

With the exception of dehydration or secondary infection, the CBC count, blood chemistry, urinalysis, and CSF analysis are usually within normal limits in canine and feline botulism. Thoracic radiographs may reveal a megaesophagus and aspiration pneumonia. Electromyographic (EMG) findings may indicate lower motor neuron disease in clinically ill animals. Decreases in the amplitudes of compound muscle action potentials and motor unit potentials are often detected. Furthermore, fibrillation potentials and decreases in nerve conduction velocity may also be detected. In order to make a definitive diagnosis, toxin must be identified in serum, vomitus or gastric contents, feces, or food samples from animals showing clinical signs. The gold standard MBA appears to have adequate sensitivity for the detection of toxin in canine and feline biological samples or in carrion. Note that the isolation of *C. botulinum* bacteria through cultures of feces, GI contents, or viscera is not a definitive diagnosis because this bacterium can be isolated from the GI tract and viscera of healthy dogs.

Differential diagnoses for canine botulism should include tick paralysis, polyradiculoneuritis (coonhound paralysis), myasthenia gravis, coral snake envenomization, and the dumb form of rabies. Both the lower motor neuron deficits and EMG findings are similar to those of tick paralysis and polyradiculoneuritis; however, due to its action on cholinergic terminals, botulism also causes cranial nerve and autonomic deficits. The nature of botulism outbreaks to affect multiple animals further differentiates the disease from other causes of lower motor neuron dysfunction.

Treatment of canine botulism consists mainly of supportive care (Critchley, 1991; Barsanti, 2006). If the

ingestion of toxin-contaminated food has been recent, gastric lavage, cathartics, and enemas may be used to decrease toxin absorption from the GI tract. However, as in other species, magnesium sulfate should be avoided. Supplemental fluids should be administered as needed to maintain hydration. Nutritional support via orogastric or parenteral administration may also be needed. Animals should be monitored for aspiration pneumonia due to megaesophagus and decreased gag reflexes. If constipation develops, enemas and stool softeners may be administered. Manual expression of the bladder may be required to decrease the occurrence of urinary tract infections. Topical ophthalmic ointments should be used to prevent corneal ulcers, which may result from diminished palpebral tone and tear production. Adequate bedding and frequent repositioning are necessary to prevent the development of decubital ulcers. In cases in which respiration is compromised, mechanical ventilation may be necessary. Antimicrobial therapy may be needed for secondary infections; however, as in other species, aminoglycosides, tetracycline, procaine penicillin, metronidazole, aminopyridines, and guanidines should be avoided.

Administration of the equine antitoxin in small animals is controversial. By the time clinical signs are noted, antitoxin is likely to be ineffective because most of the toxin is already bound to the nerve cell or has translocated into the neuron. Only the trivalent antitoxin vaccine for serotypes A, B, and E is available in the United States; this antitoxin is less useful for dogs and cats, which are usually affected by serotype C (Barsanti, 2006). However, the heptavalent antitoxin is available in other countries (Byrne and Smith, 2000; Arnon *et al.*, 2001). Because adverse reactions to antitoxin may occur, and patients with mild disease often recover with supportive care alone, antitoxin administration is usually reserved for severe cases.

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# Enterotoxins

Larry J. Thompson

## INTRODUCTION

The term food poisoning can be used to describe the result of eating pathogenic organisms or toxins in contaminated food. The list of causative agents for food-borne illness can be quite lengthy, including infectious agents (e.g., bacteria, viruses, and parasites), natural toxins (e.g., bacterial toxins, mycotoxins, shellfish poisons, and plant poisons), and other contaminants (Mead *et al.*, 1999). Veterinarians and owners often use the term garbage intoxication or garbage poisoning to describe the situation in which animals, especially dogs, eat discarded or otherwise poorly preserved foodstuffs and subsequently develop a syndrome composed mainly of severe vomiting and diarrhea. This chapter is focused on the effects of enterotoxins, which are defined as those bacterial exotoxins that are specific for the intestinal tract causing vomiting, diarrhea, and abdominal pain. Although many organisms produce enterotoxins (Vaishnavi, 1996; Fasano, 2002), those produced by *Staphylococcus aureus* and *Bacillus cereus* are stressed in this chapter. Botulism is discussed separately in the previous chapter. In contrast to enterotoxin, the term endotoxin is used to describe a cell-associated bacterial toxin, usually a lipopolysaccharide complex that is found on the outer membrane of gram-negative bacteria. Endotoxins essentially remain associated with the cell wall until the destruction of the bacteria by autolysis, external lysis, or phagocytic digestion. Although all animals can be affected by endotoxin, the horse is especially prone to disease complications due to endotoxemia (Werners *et al.*, 2005).

## BACKGROUND

This chapter focuses on the most common situations associated with enterotoxin exposures in veterinary medicine, namely dogs ingesting garbage, carrion, or other spoiled foodstuffs. Dogs readily consume a wide variety of such material, and owners often are unaware when their animal has had access to or has consumed foodstuffs of doubtful origin. Many owners have the mistaken opinion that dogs (and other animals) are not susceptible to food poisoning and thus may feed their pets foods that humans would rather not ingest. Discarded foodstuffs are often high in proteins and carbohydrates and serve as excellent substrates for the rapid growth of bacteria, often with enterotoxin release. Instances of garbage intoxication increase during warm weather and during major holidays when increased food is prepared and discarded. Under conditions of warm temperatures and adequate moisture, these discarded foodstuffs can have an almost explosive growth of bacteria, especially *S. aureus* and *B. cereus*, which are the most common causes of enterotoxin-related food poisonings in the human. Although a great deal of time and energy has been expended by public health officials in the pursuit of the origin and control of food poisonings in humans, there is a paucity of specific information concerning animals.

## PHARMACOKINETICS AND MECHANISM OF ACTION

*Staphylococcus aureus* is a facultative anaerobic gram-positive coccus that may be single, paired, or in a



grape-like cluster. *Staphylococcus aureus* does not form spores and thus contamination may be avoided by proper heat treatment of food to kill the bacteria. *Staphylococcus aureus* is able to grow in a wide range of temperatures (7–48.5°C), with an optimum range from 30 to 37°C. *Staphylococcus aureus* can also grow over a wide pH range (4.2–9.3) and can tolerate sodium chloride concentrations of up to 15%. These characteristics allow it to grow in a wide variety of foodstuffs and in situations of discarded food. *Staphylococcus aureus* is an important infective pathogen as well and can easily be found in the nostrils and on the skin of most mammals (Le Loir *et al.*, 2003). At least 14 different staphylococcal enterotoxin (SE) types have been found, and they are best described as short proteins secreted by the cell into the growth matrix (Balaban and Rasooly, 2000). All SE types are water soluble, very heat resistant, and resist most proteolytic enzymes, such as trypsin and pepsin, which make it possible for them to travel through the digestive tract to their site of action. Thus, all SE types resist the conditions that could easily destroy the bacteria that produced them. SEs are thought to have a direct effect on the intestinal epithelium and on the vagus nerve to cause stimulation of the emetic center as well as increasing peristalsis. Foods that are frequently associated with staphylococcal food poisoning include meat and meat products; poultry and egg products; salads such as egg, tuna, chicken, potato, and macaroni; bakery products such as cream-filled pastries; and milk and other dairy products. An example of a proverbial high-risk food is the potato salad at a summer picnic left out for several hours without refrigeration.

*Bacillus cereus* is in the family Bacillaceae, which are all gram-positive rod-shaped bacteria that form endospores. The family has two main divisions: the anaerobic spore-forming bacteria of the genus *Clostridium* and the aerobic or facultatively anaerobic spore-forming bacteria of the genus *Bacillus*. *Bacillus cereus* is a primary inhabitant of soils and contaminates almost all agricultural products. It is also routinely involved in the contamination and spoilage of food products. *Bacillus cereus* can also be involved in wound, eye, or systemic infections. *Bacillus cereus* food poisoning is generally described as having two types of illness caused by different metabolites. The diarrheal type of illness is caused by one or several heat-labile, high-molecular-weight proteins, whereas the vomiting (emetic) type of illness is believed to be caused by a low-molecular-weight, heat-stable peptide that has been named cereulide (Schoeni and Wong, 2005). In the human, the diarrheal syndrome is thought to be the consequence of a food-borne infection with enterotoxigenic *B. cereus* following the observation that the diarrheal enterotoxins are produced during the vegetative growth of *B. cereus* in the small intestine. *Bacillus cereus* food poisoning has been classically associated with fried rice

and other cooked rice dishes because the bacteria is frequently present in uncooked rice and heat-resistant spores may survive cooking. If cooked rice is subsequently held at room temperature, vegetative forms multiply, and a heat-stable toxin is produced that can survive brief heating, such as stir frying. *Bacillus cereus* food poisoning can also be associated with meat- or vegetable-containing foods after cooking where the food was held above room temperature for a prolonged period of time.

## TOXICITY

The symptoms of staphylococcal food poisoning occur in humans when as little as 100ng of enterotoxin is ingested (Bennett, 2005). There is a paucity of information on the dose of individual enterotoxins that will cause clinical signs in common domestic animals. The first and most common clinical sign in dogs is vomiting, which usually occurs within 2 or 3h following ingestion. This can often suffice to remove enough contaminated food from the gastrointestinal tract to prevent the development of more severe clinical signs. However, vomiting can be protracted and lead to fluid and electrolyte abnormalities. Diarrhea can often develop within 2–48h following ingestion of contaminated food and can be severe, sometimes bloody. The combination of both vomiting and diarrhea in the affected animal can quickly lead to profound fluid and electrolyte abnormalities. The animal may exhibit tenderness to the abdomen or the stomach and the intestinal tract can be distended with gas (Coppock and Mostrom, 1986).

The emetic form of *B. cereus* food poisoning is characterized in the human by an acute attack of nausea and vomiting, which occurs within 1–5h after consumption of contaminated food. Diarrhea is not a common feature in this type of illness. In the human, the diarrheal syndrome is characterized by abdominal pain, abdominal cramping, and diarrhea that often can be very watery. There is an incubation period of 4–16h, and clinical signs can persist for 12–24h. Nausea may accompany the diarrhea, but vomiting rarely occurs.

## TREATMENT

It is important to remember three factors when a food-borne problem is included in the differential diagnosis: the presenting clinical signs, the possible exposure to foodstuffs not normally included in the animal's diet, and the interval between exposure and onset of clinical problems. Some of these may not be known at the time of initial presentation but should be explored as the case is developed.

In general, treatment of garbage intoxication in animals should be directed at correcting the fluid and electrolyte abnormalities along with considerations of acid–base balance. As mentioned previously, non-complicated cases will often resolve within hours of presentation with only supportive care. However, because the clinical signs can be similar to those of more serious conditions, a more thorough diagnostic workup (e.g., survey abdominal radiographs) and close observation should always be considered. Because cases most often present following several vomiting episodes, the use of emetics is usually not indicated. In the case of protracted vomiting, the judicious use of antiemetics should be considered. Administration of activated charcoal is not required for known garbage intoxications, but many clinicians will use it as part of a general approach to these cases. Activated charcoal should not be given to dehydrated animals unless fluid administration is also initiated. Antibiotics are not indicated in uncomplicated cases of garbage intoxication.

## CONCLUDING REMARKS

This chapter describes garbage intoxication with emphasis on preformed enterotoxins as the causative agents.

The great number of other types of food-borne illnesses, including infectious agents and toxins, require other diagnostic and treatment considerations.

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# Cyanobacterial (blue-green algae) toxins

Birgit Puschner and Amber Roegner

## INTRODUCTION

Freshwater cyanobacterial blooms hold the potential to significantly impact the health of both animal and human populations utilizing surface waters for drinking water, daily living, and recreation worldwide. In addition to the often visually stunning nature of these proliferations of cyanobacterial species, the blooms can result in the production of a variety of compounds, from malodorous ones that affect the taste of the water to dermal and gastrointestinal irritants and severe neurotoxins, gastrointestinal toxins, and hepatotoxins. Among the 2000 species identified through morphological criteria, more than 80 are known to be toxigenic, and as assays for detection and toxicity continue to improve, this number will continue to grow. George Francis first reported a toxigenic bloom in the journal *Nature* in 1878. He reported a “poisonous Australian lake” with “a thick scum like green oil paint” and vividly described acute intoxications of sheep, horses, dogs, and pigs. Analysis of archaeological evidence coupled with evolving understanding of modern blooms have begun to implicate the role of cyanotoxin poisoning in more widespread mammalian die-offs dating back to the Pleistocene age (i.e., approximately 150,000 years BC; Braun and Pfeiffer, 2002), and even a controversial hypothesis about the role of cyanobacteria in the various mass extinction events has begun to emerge (Castle and Rodgers, 2009).

Since Francis’ publication in 1878, numerous case reports describing animal morbidity and mortality after exposure to cyanotoxins have been published (Fitzgerald and Poppenga, 1993; Naegeli *et al.*, 1997; Puschner *et al.*, 1998, 2008, 2010; Gugger *et al.*, 2005; Nasri *et al.*, 2008;

Wood *et al.*, 2010). The frequency of blue-green algae poisoning in animals is likely underreported due to lack of methods to confirm exposure; in addition, geographical distribution of these case reports is likely biased by available resources. Diagnostic confirmation of suspect blue-green algae poisoning cases of humans and animals requires extensive effort from both toxicologists and clinicians, and resources are often not readily available. New algal toxins are continuously being discovered, and oral bioavailability and toxicity data are often unavailable. It is probable that blue-green algae poisonings are more common in animals than in humans due to animals’ greater direct dependence and contact with surface waters.

Pursuant to several major human intoxications in Australia, Europe, and Brazil (Falconer and Humpage, 2005), in 1998 the World Health Organization (WHO) proposed a guidance value for the maximum permissible concentration in potable water sources for microcystin-LR, the most commonly reported cyanotoxin worldwide. Water sanitation agencies in many countries in Europe, North America (Canada), South America (Brazil), and Oceania (Australia and New Zealand) adopted these guidelines. In addition, as a result of a tragedy in 1996 in which more than 100 patients at a hemodialysis clinic received inadequately treated drinking water (Azevedo *et al.*, 2002), most of whom developed acute liver failure as a result of being exposed to cyanotoxins, Brazil has adopted more comprehensive and stringent guidelines to include other cyanotoxins (Burch, 2008). Awareness of imminent health risks for wild and domestic terrestrial vertebrates has increased during approximately the past decade, in part due to veterinary case reports; however, the extent and

heterogeneity of the impact are still far from understood. Veterinarians thus have the opportunity to substantially deepen the understanding of the impact of these cyanotoxins on animal and human populations alike.

## BACKGROUND

Nutrient-rich runoff into surface waters – particularly nitrogen- and phosphorus-rich fertilizers, soaps, and waste products – has led to significant eutrophication worldwide (>40% in Europe, Asia, and America) (Bartram *et al.*, 1999; Smith, 2003). As a major consequence of shifting nutrient additions, previously nutrient-limited photosynthetic microorganisms proliferate. Depending on the limitations of the system and the types of nutrients added, a few species (generally one or two) outcompete the others, thereby considerably reducing the heterogeneity of the phytoplankton community. In such conditions, cyanobacteria often predominate through adaptive processes, and substantial shifts in the microscopic and macroscopic food web may occur. Anoxic conditions can also result in fish kills, and falling debris from blooms can have profound impacts on the invertebrates in the sediment below (Pearl *et al.*, 2001; Havens, 2008).

Among the oldest microorganisms, these oxygenic photosynthetic prokaryotes may be organized as individual cells (e.g., *Synechococcus*), filaments (e.g., *Planktothrix*), or colonies (e.g., *Microcystis*). More than 2000 cyanobacterial species belong to four orders based on morphological and morphometric criteria in botanical code (Anagnostidis and Komarek, 1985); however, classification based on bacterial code defines five sections through combined use of genetic data, morphological criteria, and cellular fission (Rippka *et al.*, 1979).

Both pelagic (suspended in the water column) and benthic (along the bottom) cyanobacteria can proliferate into blooms. Pelagic blooms, which are easier to visually detect because of the evident scum formation at the surface, usually occur in mesotrophic and eutrophic ecosystems (concentrations in phosphorus >30 µg/L), during the summer, in water temperatures greater than 20°C, and in low turbulence. Proliferations of benthic species generally occur during the summer on the surfaces of sediments, stones, or macrophytes in small oligotrophic rivers or in oligotrophic lakes (Mez *et al.*, 1997).

In the past, most cases were diagnosed by positive identification of the algae in the suspect water source along with the occurrence of consistent clinical signs and pathological findings. However, new analytical methods can now be applied to detect toxins in biological specimens of animals or humans with suspect exposure to toxic algal blooms Figure 72.1 illustrates the wide variety

in chemical structures of cyanotoxins and the need for specific detection methods. (Yuan *et al.*, 2006; Humbert, 2010). These capabilities will allow for in-depth diagnostic investigations and a better estimate of the true frequency of blue-green algae poisonings in livestock, pets, and wildlife. Table 72.1 provides an overview of cyanobacterial species known to produce a large number of toxins. Some species can produce a variety of cyanotoxins and thus it is difficult to predict the nature and the level of the toxin production during a bloom event. This chapter focuses on the several types of cyanotoxins known to have the greatest impact on veterinary species and presents the current understanding of their toxic mechanisms, toxicokinetics, and diagnostic and therapeutic approaches with a focus on veterinary medicine.

## MICROCYSTINS

Produced by multiple cyanobacteria, including species within the genera *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc*, *Oscillatoria*, and *Anabaenopsis*, microcystins have been detected worldwide (Fromme *et al.*, 2000; Hitzfeld *et al.*, 2000; Ballot *et al.*, 2004; Briand *et al.*, 2005; Karlsson *et al.*, 2005a; Ndeti and Muhandiki, 2005; Agrawal *et al.*, 2006). Not all strains are capable of producing microcystins. In recent years, a useful diagnostic tool to test for the presence of toxin-producing genes has emerged (Hisbergues *et al.*, 2003). Although the reason for production is not understood, environmental factors, such as pH, nutrient concentrations, and water temperature, clearly trigger production, increasing with water temperature, elevated concentrations of phosphorus and nitrogen, iron limitation, and globally with the growth rate (Briand *et al.*, 2005; Downing *et al.*, 2005; Sevilla *et al.*, 2008). Microcystin concentrations may be highest when the growth of the cyanobacteria is high, but toxin concentrations do not necessarily correlate with cell count, and toxins may occur any time of the year. Although predominantly found in freshwater, microcystin-producing blooms have also been described in saline ecosystems (Atkins *et al.*, 2001; Carmichael and Li, 2006).

Potent cyclic heptapeptides causing acute hepatotoxicosis in mammals, microcystins have also been demonstrated to be toxic to reptiles, amphibians, and aquatic species, as well as invertebrates and even plant species (McElhiney *et al.*, 2001; Malbrouck and Kestemont, 2006; Nasri *et al.*, 2008; Amado and Monserrat, 2010). In freshwater, the toxins are retained inside the cyanobacteria and only released upon cell damage, lysis, and death; destruction of algal mats (either naturally or through the application of herbicides) may result in a pulse of microcystin release following destruction of the individual cell walls. After oral exposure to microcystin-containing



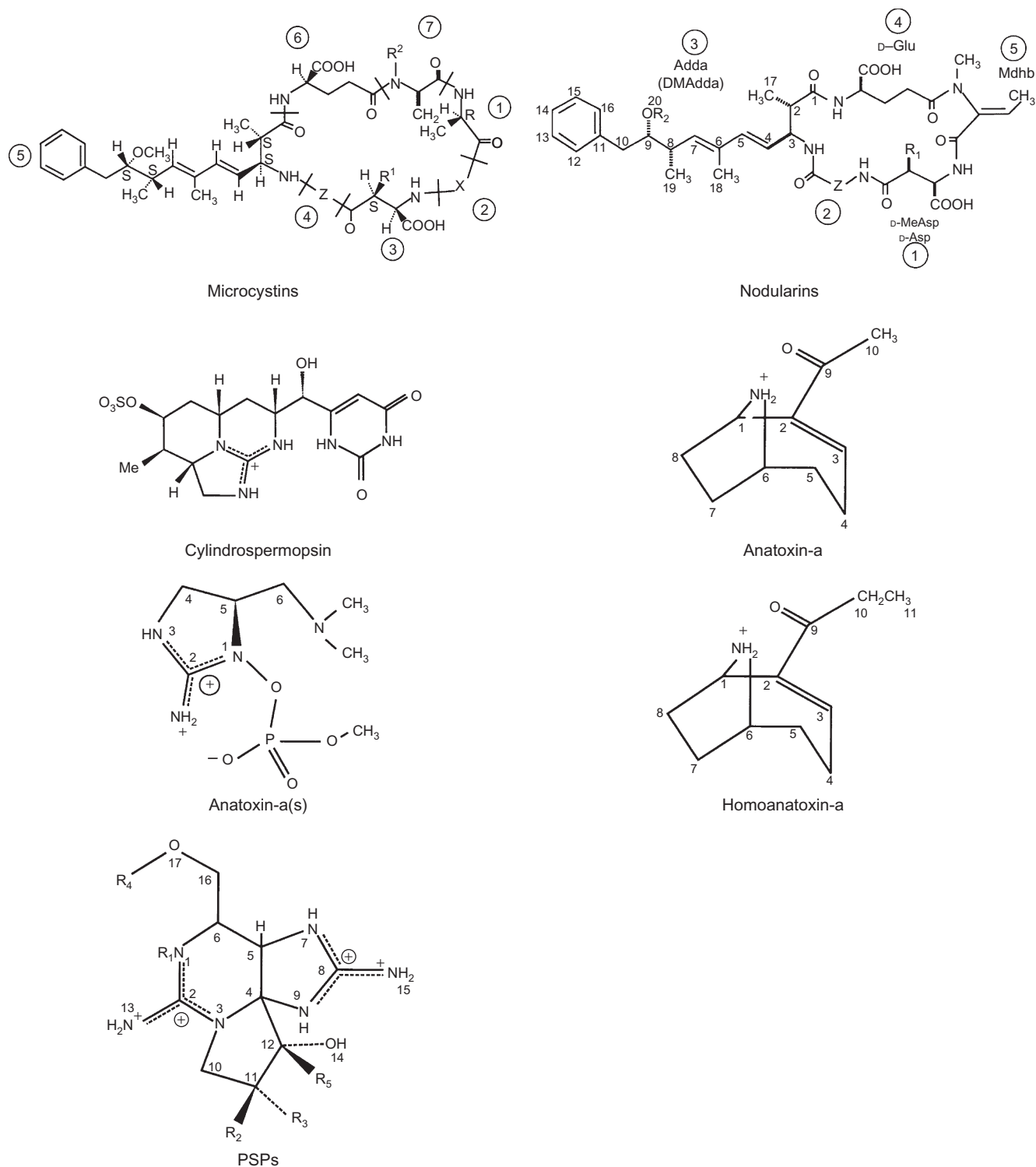


FIGURE 72.1 Structural formulas of cyanobacterial toxins.

algae, the acidic environment of the stomach can result in the release of microcystins. Commercially available blue-green algae food supplements also present a potential route of oral exposure (Schaeffer *et al.*, 1999; Dietrich and Hoeger, 2005).

More than 80 different structural variants of microcystins have been identified from various genera of cyanobacteria (Luukkainen *et al.*, 1994; Lawton *et al.*, 1995; Welker and von Döhren, 2006). The shared structure involves an amino acid called ADDA

TABLE 72.1 Potential toxins produced by the different cyanobacterial species

Cyanobacterial species	Toxins	Cyanobacterial species	Toxins
<i>Anabena bergii</i>	Cylindrospermopsin	<i>Nodularia spumigena</i>	Nodularins
<i>Anabaena circinalis</i>	Anatoxin-a, saxitoxins, microcystins	<i>Nostoc linckia</i>	Microcystins
<i>Anabaena flos-aquae</i>	Anatoxins, microcystins	<i>Nostoc muscorum</i>	Aplysiatoxin
<i>Anabaena lappoinica</i>	Cylindrospermopsin	<i>Nostoc paludosum</i>	Microcystins
<i>Anabaena lemmermanni</i>	Microcystins, anatoxin-a(s)	<i>Nostoc rivulare</i>	Microcystins
<i>Anabaena macrospora</i>	Anatoxin-a	<i>Nostoc zetttriedrii</i>	Microcystins
<i>Anabaena medotae</i>	Anatoxin-a	<i>Nostoc</i> sp.	Microcystins
<i>Anabaena planctonica</i>	Anatoxin-a	<i>Oscillatoria formosa</i>	Homoanatoxin-a
<i>Anabaena spiroides</i>	Anatoxin-a, microcystins	<i>Oscillatoria limosa</i>	Microcystins
<i>Anabaena</i> sp.	Anatoxin-a	<i>Oscillatoria perornata</i>	Microcystins
<i>Anabaenopsis milleri</i>	Microcystins	<i>Oscillatoria tenuis</i>	Microcystins
<i>Aphanizomenon flos-aquae</i>	Anatoxin-a, saxitoxins, cylindrospermopsin	<i>Oscillatoria nigroviridis</i>	Oscillatoxin-a
<i>Aphanizomenon gracile</i>	Saxitoxins	<i>Oscillatoria</i> sp.	Anatoxin-a
<i>Aphanizomenon issatschenkoi</i>	Saxitoxins, anatoxin-a	<i>Phormidium favosum</i>	Anatoxin-a
<i>Aphanizomenon ovalisporum</i>	Cylindrospermopsin	<i>Planktothrix agardhii</i>	Microcystins, anatoxin
<i>Aphanizomenon</i> sp.	Anatoxin-a	<i>Planktothrix isothrix</i>	Microcystins, anatoxin
<i>Arthrospira fusiformis</i>	Anatoxin-a, microcystin-YR	<i>Planktothrix mougeotii</i>	Microcystins
<i>Calothrix crustacean</i>	Aplysiatoxin	<i>Planktothrix rubescens</i>	Microcystins
<i>Coelosphaerium kuetzingianum</i>	Neuro-/hepatotoxins	<i>Planktothrix</i> sp.	Anatoxin-a
<i>Cyanobium bacillare</i>	Microcystins	<i>Pseudanabaena</i> sp.	Neurotoxin
<i>Cylindrospermopsis raciborskii</i>	Cylindrospermopsin, saxitoxins	<i>Radiocystis fernandoi</i>	Microcystins
<i>Cylindrospermum</i> sp.	Anatoxin-a	<i>Radiocystis curvata</i>	Cylindrospermopsin
<i>Fisherella epiphytica</i>	Neuro-/hepatotoxins	<i>Raphidiopsis curvata</i>	Cylindrospermopsin
<i>Geitlerinema acutissimum</i>	Dermatotoxins	<i>Raphidiopsis mediterranea</i>	Homoanatoxin-a
<i>Geitlerinema pseudacutissimum</i>	Dermatotoxins	<i>Schizothrix calcicola</i>	Aplysiatoxins
<i>Gloeotrichia echinulata</i>	Neuro-/hepatotoxins	<i>Scytonema mirabile</i>	Antimicrobial
<i>Hapalosiphon fontinalis</i>	Neuro-/hepatotoxins	<i>Scytonema ocellatum</i>	Scytophycin
<i>Hapalosiphon hibernicus</i>	Microcystins	<i>Scytonema pseudohofmannii</i>	Scytophycin
<i>Hormothamnion enteromorphoides</i>	Cytotoxin, hepatotoxins	<i>Schizothrix calcicola</i>	Aplysiatoxins
<i>Limnospira redekei</i>	Microcystins	<i>Scytonema hofmanni</i>	Scytophycins a and b
<i>Lyngbya gracilis</i>	Debromoaplysiatoxin	<i>Scytonema pseudohofmanni</i>	Scytophycins a and b
<i>Lyngbya majuscula</i>	Lyngbyatoxin-a	<i>Snowella lacustris</i>	Microcystin
<i>Lyngbya wollei</i>	Saxitoxins	<i>Stigonematales</i> sp.	BMAA
<i>Microcystis aeruginosa</i>	Microcystins	<i>Symploca muscorum</i>	Aplysiatoxin
<i>Microcystis botrys</i>	Microcystins	<i>Synechococcus</i> sp.	Hemolysins
<i>Microcystis farlowian</i>	Ichtyotoxin	(marine species)	
<i>Microcystis flos-aquae</i>	Microcystins	<i>Synechocystis</i> spp.	Anatoxin-a, microcystins
<i>Microcystis panniformis</i>	Microcystins	<i>Tolypothrix byssoidea</i>	Cytotoxin
<i>Microcystis viridis</i>	Microcystins, microviridin	<i>Trichodesmium erythraeum</i>	Neurotoxin
<i>Microcystis wesenbergii</i>	Microcystins	<i>Trichodesmium thiebautii</i>	Microcystins, neurotoxins
<i>Microcystis</i> sp.	Anatoxin-a	<i>Trichormus variabilis</i>	Anatoxin-a
		<i>Umezakia natans</i>	Cylindrospermopsin
		<i>Woronichinia naegeliana</i>	Anatoxin-a

(3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca; 4,6-dienoic acid) and six other amino acids, including two variable positions that designate the congener. The most extensively studied, microcystin-LR, has been found worldwide and has caused acute, lethal hepatotoxicoses in farm animals (Carbis *et al.*, 1994; Mez *et al.*, 1997; Puschner *et al.*, 1998) and hepatic injury in humans (Azevedo *et al.*, 2002; Rao *et al.*, 2002).

### Pharmacokinetics/toxicokinetics

Despite the abundant literature on microcystins, understanding of pharmacokinetics remains limited,

particularly with regard to potential species variations. Most studies have been conducted in mice after intravenous (i.v.) and intraperitoneal (i.p.) administration of cyanobacteria, their filtrates, and, in some instances, purified microcystins. After i.v. and i.p. administration in mice and rats, microcystins are rapidly distributed to the liver (Falconer *et al.*, 1986; Robinson *et al.*, 1991). Plasma half-lives of microcystin-LR in mice after i.v. administration were 0.8 and 6.9 min for the alpha and beta phases of elimination (Robinson *et al.*, 1991). Interestingly, the hepatic concentration of <sup>3</sup>H-microcystin-LR remained constant throughout the 6-day study, indicating accumulation in this target organ. This study also demonstrated that approximately 9 and 14% of the dose was

excreted in urine and feces, respectively, after 12 h, with 60% of it being excreted unchanged. Additional studies in swine have also indicated that the majority is excreted unchanged, with only two metabolites detected; biliary excretion is also noted after less than 1 h of i.v. administration (Stotts *et al.*, 1997). The exact route of metabolism is yet to be defined, but glutathione and cysteine conjugation have been identified and may represent major detoxification pathways (Kondo *et al.*, 1996; Pflugmacher *et al.*, 1998). Other metabolites have been identified *in vivo* and *in vitro*, but further work is needed to define their roles.

Data on bioavailability for microcystins are needed to better evaluate risk from oral ingestion. Absorption occurs in the small intestine (Ito *et al.*, 2000); thus, the integrity of the intestinal mucosa can significantly impact the degree of absorption (Zeller *et al.*, 2011). Altered cell permeability of the small intestine in aged mice lends them more susceptible than young animals (Ito *et al.*, 1997). Once absorbed, microcystins are rapidly distributed to the liver (Runnegar *et al.*, 1981; Fischer *et al.*, 2000), but they can also reach lung, heart, and capillaries (Ito *et al.*, 2000). Based on radiolabeled experiments in which microcystins were administered i.v. in Wistar rats, uptake into the kidney appears to be important for excretion. Results of uptake experiments with radiolabeled dihydro-MC-LR demonstrated that OATP1B1 (organic anion transporter protein) and OATP1B3 are involved in uptake of MC-LR (Seithel *et al.*, 2007). Studies in fish (i.v.) have demonstrated that the liver is the primary target, followed by kidney and gonads, although uptake does occur into muscle tissue and cardiovascular effects are observed (Malbrouck and Kestemont, 2006). Absorption of microcystin via the respiratory route (Ito *et al.*, 2001) has been demonstrated to lead to lethality in mice and also induce damage to nasal epithelium at lower exposures (Benson *et al.*, 2005), thus posing a threat from aerosolization of the compound.

## Mechanism of action

Specifically toxic to liver, microcystins cause severe hepatomegaly macroscopically and progressive centrilobular hepatocyte rounding, dissociation, and necrosis microscopically. Breakdown of the sinusoidal endothelium and intrahepatic hemorrhage ultimately result in death (Hooser *et al.*, 1991a; Falconer and Yeung, 1992). Unable to permeate cell membranes, microcystins enter hepatocytes via the bile acid transporter mechanism (Hooser *et al.*, 1991b). Once inside the hepatocytes, microcystins are potent inhibitors of protein phosphatases 1 and 2A (Falconer and Yeung, 1992; Runnegar *et al.*, 1993). The disruption of the cytoskeletal components and the associated rearrangement of filamentous

actin within hepatocytes account for the morphological changes, although other mechanisms play a role in the development of liver lesions. Microcystins induce apoptosis of hepatocytes via induction of free radical formation and mitochondrial alterations (Ding and Ong, 2003). A single-dose i.v. in rats demonstrated an increase in liver sphingolipid levels at higher doses (implicating ceramide-mediated apoptosis), a dose-dependent decreased PP2A expression, and ultimately a dose-dependent decreased expression of Bcl2 family proteins, involved in cell cycle/apoptosis regulation (Billam *et al.*, 2008). The role of oxidative stress has become increasingly apparent, and the ultimate toxic effect may depend on the ability of antioxidant pathways to counter the stressors (Ding and Ong, 2003; Jayaraj *et al.*, 2006; Xiong *et al.*, 2010). In addition, microcystins are classified as tumor-promoting compounds (Humpage and Falconer, 1999). Investigations have indicated the role of protooncogenes in this tumorigenesis, hypothesized to be a sequelae of dysregulation of phosphorylation (Li *et al.*, 2009). Several studies have demonstrated the ability of microcystins to induce DNA damage in liver cells (Zegura *et al.*, 2011).

Clinical signs of microcystin poisoning have been described in a number of reports in livestock, humans, and wildlife in the United States (DeVries *et al.*, 1993; Fitzgerald and Poppenga, 1993; Galey *et al.*, 1987; Puschner *et al.*, 1998) and other countries (Done and Bain, 1993; Van Halderen *et al.*, 1995; Mez *et al.*, 1997; Naegeli *et al.*, 1997; Azevedo *et al.*, 2002; Ballot *et al.*, 2004; Ndeti and Muhandiki, 2005; Handeland and Østensvik, 2010; Wood *et al.*, 2010). Interestingly, laboratory animals select water with microcystin-producing strains of cyanobacteria over a water source with nontoxic strains (Lopez Rodas and Costas, 1999), suggesting an increased risk for toxicosis in animals due to behavioral preferences. Microcystin intoxication should be suspected in cases of acute hepatotoxicosis with clinical signs of diarrhea, vomiting, weakness, pale mucous membranes, and shock. Although most animals die within a few hours of exposure, some animals may live for several hours and develop hyperkalemia, hypoglycemia, nervousness, recumbency, and convulsions. Animals that survive the acute intoxication may develop hepatogenous photosensitization. Nephrotoxic effects have been described in laboratory animals after chronic microcystin exposure (Milutinovic *et al.*, 2003). Evidence suggests potential suppression of immune function at sublethal exposures (Shi *et al.*, 2004). In humans, primary liver cancer as well as colorectal cancer have been associated with microcystin-contaminated drinking water (Ueno *et al.*, 1996; Zhou *et al.*, 2002). In mice, subchronic exposure i.p. of microcystin-LR (20 µg/kg) causes the appearance of hepatic nodules, a characteristic not observed after oral subchronic administration (Ito *et al.*, 1997).

## Toxicity

The lethal doses 50 ( $LD_{50}$ s) for microcystins vary between 50  $\mu\text{g/kg}$  and 11  $\text{mg/kg}$ , depending on the microcystin analog, the species affected, and the route of administration. In mice, the oral  $LD_{50}$  value for microcystin-LR is 10.9  $\text{mg/kg}$ , whereas the i.p.  $LD_{50}$  is 50  $\mu\text{g/kg}$ . Because most blooms contain a number of structural variants of microcystins, it is difficult to estimate the toxicity potential of a bloom. The no-observed-adverse-effect level for orally administered microcystin LR to mice is 40  $\mu\text{g/kg/day}$  (Fawell *et al.*, 1994). In pigs, the lowest-observed-adverse-effect level for microcystin-LR is 100  $\mu\text{g/kg/day}$  (Falconer *et al.*, 1994), and in rat it is 50  $\mu\text{g/kg/day}$  (Heinze, 1999). WHO set the tolerable daily intake (TDI) for human ingestion of microcystin-LR at 0.04  $\mu\text{g/kg/day}$  (Kuiper-Goodman *et al.*, 1999). The potential risk to humans by ingesting food products derived from animals exposed to microcystins was evaluated in beef (Orr *et al.*, 2003) and dairy cattle (Orr *et al.*, 2001). Based on these studies, it is unlikely that consumption of milk, meat, or liver poses a significant health risk to humans. It might be prudent to establish specific guidelines for nonlethal, chronic microcystin exposure in livestock.

## Treatment

No specific antidote for microcystins exists. The rapid onset of acute hepatotoxicosis renders therapeutic intervention quite difficult, and mortality rates are very high. In addition, despite the evaluation of numerous treatment options, no specific therapy has been proven to be effective. The most promising strategy appears to be prevention of uptake into hepatocytes through the administration of compounds that may compete for the specific transporters associated with microcystin uptake; administration of the antibiotic rifampin (i.p.) in mice effectively reduced mortality after exposure (i.p.) to microcystin-LR (Hermansky *et al.*, 1991). By contrast, other compounds, such as glutathione, silymarin, and cyclosporine A, were only beneficial if administered as a prophylactic (Hermansky *et al.*, 1991; Rao *et al.*, 2004). These compounds may help reduce microcystin toxicity in chronic exposure scenarios. Due to the role of oxidative stress, antioxidants such as vitamin E, selenium, and green tea polyphenols also appear to be beneficial prophylactically (Gehring *et al.*, 2003a,b; Jayaraj *et al.*, 2007; Xu *et al.*, 2007). Although the adsorption of microcystins by activated charcoal was used successfully to decontaminate drinking water (Warhurst *et al.*, 1997), this decontamination procedure was not protective in mice dosed with microcystins (Mereish and Solow, 1989); no data are available for other species.

Diagnosis of microcystin toxicosis is corroborated by identification of microcystin-containing water in the environment of the animal. Identification of algae material in water and gastric contents is an important component of the diagnostic workup but does not confirm intoxication. As described previously, the toxicity of the cyanobacteria is strain specific, and morphological observations alone cannot predict the hazard level, but polymerase chain reaction detection of microcystin-producing genes can help identify a potentially culpable species. Detection of microcystins in gastric contents is confirmatory, but these tests are not routinely available at diagnostic laboratories and are limited to a few structural variants. In the past, the mouse bioassay was used to determine the toxicity of crude algal biomass in suspicious blue-green algae poisonings. Although many assays are available to analyze water samples for microcystins (Maizels and Budde, 2004; McElhiney and Lawton, 2005; Frias *et al.*, 2006), there are only limited methods available to reliably and accurately detect microcystins in biological specimens collected from animals suspected to have died from microcystin intoxication (Bogialli *et al.*, 2005; Karlsson *et al.*, 2005b; Chen *et al.*, 2009). An electrospray ionization liquid chromatography–mass spectrometry method has been developed to determine the bound microcystin concentrations in animal tissues, which provides an estimate of the total microcystin burden in exposed animals (Ott and Carmichael, 2006).

Differential diagnoses in animals with a clinical presentation of liver failure include other toxic ingestions, such as amanitins, cocklebur, cycad palm, aflatoxin, xylitol, certain heavy metals, and acetaminophen overdose. Careful evaluation of the history, feed, and environment of the animal can help eliminate most of the toxicant differentials on the list.

## ANATOXINS

Anatoxins are mainly produced by cyanobacteria in the *Anabaena* genus (Beltran and Neilan, 2000) but also by other genera, such as *Plankthrix*, *Oscillatoria*, *Microcystis*, *Aphanizomenon*, *Cylindrospermum*, and *Phormidium*. Unfortunately, specific data on factors resulting in anatoxin-a production are lacking, which makes toxin production unpredictable. Although anatoxin-a is considered unstable in the environment, certain environmental conditions are known to result in continuous toxin production. Reports of anatoxin poisoning are less frequent than those of microcystin toxicosis; however, poisoning has occurred worldwide (Edwards *et al.*, 1992; Gunn *et al.*, 1992; Beltran and Neilan, 2000;



Fromme *et al.*, 2000; Gugger *et al.*, 2005; Yang and Boyer, 2005; Wood *et al.*, 2007; Puschner *et al.*, 2008, 2010). Anatoxins are neurotoxins and can generally be divided into anatoxin-a, homoanatoxin-a, and anatoxin-a(s). Anatoxin-a is a secondary amine and has been detected in blooms worldwide. Homoanatoxin-a is a methyl derivative of anatoxin-a and has been identified in blooms in Japan (Namikoshi *et al.*, 2004), Ireland (Furey *et al.*, 2003), Sweden (Skulberg *et al.*, 1992), and New Zealand (Wood *et al.*, 2007). Anatoxin-a(s) is a unique N-hydroxyguanidine methyl phosphate ester that has been detected in the Americas (Monserrat *et al.*, 2001) and Europe (Henriksen *et al.*, 1997). Commercially available blue-green algae dietary supplements also represent a potential source (Rellán *et al.*, 2009).

### Pharmacokinetics/toxicokinetics

Definite data on the toxicokinetics of anatoxin-a, homoanatoxin-a, and anatoxin-a(s) have not been established. Based on the rapid onset of clinical signs after oral exposure, rapid absorption of the toxins is suspected. Anatoxin-a has been detected in the urine and bile of a poisoned dog, confirming that anatoxin-a is, at least in part, excreted unchanged in urine and bile (Puschner *et al.*, 2010).

### Mechanism of action

Anatoxin-a is a potent cholinergic agonist at nicotinic acetylcholine receptors in neurons and at neuromuscular junctions (Thomas *et al.*, 1993). Anatoxin-a has two enantiomers, with (+)anatoxin-a having a higher binding affinity than the (–) form (Spivak *et al.*, 1980; Zhang and Nordberg, 1993). Compared to nicotine, anatoxin-a is approximately 20 times more potent than acetylcholine. After continuous electrical stimulation at the neuromuscular junctions, a nerve block may follow and result in death due to respiratory paralysis. Furthermore, anatoxin-a has modulatory action at presynaptic neuronal nicotinic acetylcholine receptors, which can lead to dopamine as well as noradrenaline release (Barik and Wonnacott, 2006; Campos *et al.*, 2010). Clinical signs of anatoxin-a poisoning include a rapid onset of rigidity and muscle tremors followed by convulsions, paralysis, respiratory failure, cyanosis, and death. Death usually occurs within minutes to a few hours. Anatoxin-a poisonings have been reported in dogs in Europe (Edwards *et al.*, 1992; Gunn *et al.*, 1992; James *et al.*, 1997; Gugger *et al.*, 2005) and the United States (Puschner *et al.*, 2008, 2010). Anatoxin-a is also considered a contributing factor in the deaths of Lesser Flamingos in Kenya (Krienitz *et al.*, 2003). Homoanatoxin-a is a methyl derivative of

anatoxin-a with similar pharmacological and toxicological properties (Wonnacott *et al.*, 1992), and it has been implicated in dog deaths in New Zealand (Wood *et al.*, 2007). In addition to being a nicotinic agonist, homoanatoxin-a can increase the release of acetylcholine from peripheral cholinergic nerves through opening of endogenous voltage-dependent neuronal L-type  $\text{Ca}^{2+}$  channels (Aas *et al.*, 1996).

Anatoxin-a(s) is different from anatoxin-a and homoanatoxin-a. This neurotoxin has a unique chemical structure and is a naturally occurring irreversible acetylcholinesterase inhibitor. The increased concentrations of acetylcholine in the synapse lead to persistent stimulation, followed by a neuronal muscular block (Cook *et al.*, 1990; Hyde *et al.*, 1991). The mechanism of toxic action is similar to that of organophosphorus and carbamate insecticides, as well as some chemical warfare nerve agents (Patocka *et al.*, 2011). However, one of the main differences is that anatoxin-a(s) acts only in the periphery, whereas the insecticides inhibit acetylcholinesterase in the brain and retina (Cook *et al.*, 1989). Animals poisoned with anatoxin-a(s) show a rapid onset of excessive salivation (“s” stands for salivation), lacrimation, diarrhea, and urination. Clinical signs of nicotinic receptor overstimulation including tremors, incoordination, convulsions, recumbency, and respiratory arrest are most commonly observed in cases with a lethal outcome. Animals often die within 30 min of exposure. Animals that die from anatoxin-a, homoanatoxin-a, or anatoxin-a(s) toxicosis do not show specific gross or microscopic lesions. Anatoxin-a(s) poisoning has been reported in pigs, birds, dogs, and calves in the United States and Europe (Mahmood *et al.*, 1988; Cook *et al.*, 1989; Onodera *et al.*, 1997). Because of the lack of specific detection methods for anatoxin-a(s), the natural occurrence of this neurotoxin has not been fully evaluated.

### Toxicity

In mice, the i.p.  $\text{LD}_{50}$  of anatoxin-a is 200  $\mu\text{g}/\text{kg}$  (Stevens and Krieger, 1991), whereas the i.v.  $\text{LD}_{50}$  is estimated to be less than 100  $\mu\text{g}/\text{kg}$ . The oral toxicity of anatoxin-a is much higher, with an oral  $\text{LD}_{50}$  in mice reported to be greater than 5 mg/kg. Several studies have shown that there are significant species differences with regard to anatoxin-a toxicity. Whereas an anatoxin-a containing *Aphanizomenon flos-aquae* bloom was toxic to sheep after i.p. administration, oral administration failed to induce toxicity (Runnegar *et al.*, 1988). In contrast, calves developed toxicity after oral administration of an anatoxin-a containing *A. flos-aquae* bloom (Carmichael *et al.*, 1977). The i.p.  $\text{LD}_{50}$  of homoanatoxin-a in mice is 250  $\mu\text{g}/\text{kg}$  (Skulberg *et al.*, 1992). Anatoxin-a(s) is much more toxic than anatoxin-a or homoanatoxin-a, with an i.p.  $\text{LD}_{50}$  in mice of 20  $\mu\text{g}/\text{kg}$  (Briand *et al.*, 2003).

## Treatment

There is no specific antidote for anatoxin-a. Because of the rapid onset of clinical signs, emesis is not likely to be useful. Although no studies have evaluated the efficacy of specific decontamination procedures, administration of activated charcoal has been recommended. In addition, artificial respiration may be of benefit along with general supportive care. Specific measures to control seizures include benzodiazepines, phenobarbital, or pentobarbital. If given, they may cause central nervous system and respiratory depression, and careful monitoring of the animal is necessary. In any seizing animal, control of body temperature is an important part of the symptomatic care.

Treatment of animals poisoned with anatoxin-a(s) is primarily symptomatic and supportive. Decontamination procedures can be considered but have not been evaluated. It has been shown that 2-PAM is not able to reactivate the inhibited acetylcholinesterase and is therefore not recommended (Hyde and Carmichael, 1991). Atropine should be given at a test dose to determine its efficacy in animals with life-threatening clinical signs. After the test dose, atropine can be given repeatedly until cessation of salivation. It is important to carefully monitor the animal for anticholinergic effects and to reduce or discontinue atropine if adverse effects develop.

As with other cyanobacteria toxins, toxicity is strain-specific, and identification of the cyanobacteria alone cannot predict the toxicity level. Therefore, detection of anatoxin-a in biological specimens is confirmatory, but these tests are not routinely available (James *et al.*, 1998; Puschner *et al.*, 2010). Anatoxin-a was confirmed in stomach content, liver, urine, and bile of dogs (Gugger *et al.*, 2005; Puschner *et al.*, 2010). In suspect cases, environmental and biological samples should be saved for toxicological and phylogenetic analysis.

Diagnosis of anatoxin-a(s) toxicosis is aided by the determination of blood acetylcholinesterase activity. However, organophosphorus and carbamate insecticides can also inhibit acetylcholinesterase, and additional diagnostic workup is needed to establish a firm diagnosis. This includes the determination of brain acetylcholinesterase postmortem (unchanged in cases of anatoxin-a(s) poisoning), screening of gastrointestinal contents for insecticides, examination of stomach contents (possible identification of cyanobacteria), and a careful evaluation of the environment (access to freshwater and access to insecticides). Detection methods for anatoxin-a(s) are rare. A biosensor method has been developed that allows the quantitation of anatoxin-a(s) in environmental samples (Devic *et al.*, 2002). New analytical methods for anatoxin-a(s) are necessary to better document the distribution of this neurotoxin in freshwater worldwide. Phylogenetic analysis of 16S rRNA gene sequences will help in the species identification.

## MISCELLANEOUS FRESHWATER CYANOBACTERIAL TOXINS

Although microcystin and anatoxin poisonings comprise the majority of cases reported in animals, other cyanotoxins are of concern. Saxitoxins and derived forms belong to the group of paralytic shellfish poisoning (PSP) toxins and have been produced by a number of freshwater cyanobacteria, including *A. flos-aquae*, *Cylindrospermopsis raciborskii*, *Anabaena circinalis*, *Lyngbya wollei*, *Planktothrix* sp., and *Aphanizomenon gracile* (Carmichael *et al.*, 1997; Kaas and Henriksen, 2000; Molica *et al.*, 2005; Ballot *et al.*, 2010). All saxitoxin analogs have high toxicity in mammals by blocking voltage-gated sodium channels, leading to respiratory arrest, neuromuscular weakness, and cardiovascular shock. Whereas intoxications of birds and cats have been associated with ingestion of PSP contaminated fish and clams (Landsberg, 2002), there is only one confirmed report of PSP toxin-associated mortality from exposure to contaminated freshwater (Negri *et al.*, 1995). Fourteen sheep showed signs of trembling, recumbency, and death in Australia after exposure to toxic concentrations of PSPs produced by *Anabaena circinalis*. Because PSP toxins are produced by several species of freshwater cyanobacteria, terrestrial animals and humans are at risk of being exposed to these toxins. Thus, it is important to reliably evaluate animals with neurologic signs after access to freshwater for possible PSP exposure because the risk certainly exists.

Another cyanotoxin, the alkaloid cylindrospermopsin, has caused deaths in cattle (Saker *et al.*, 1999) and severe gastrointestinal disease in humans. Cylindrospermopsin is a potent inhibitor of protein synthesis and can lead to various degrees of injury to the liver, kidneys, adrenal gland, intestine, lung, thymus, and heart (Griffiths and Saker, 2003). Furthermore, this cyanotoxin is of particular concern because of its mutagenic and possibly carcinogenic activities. Cylindrospermopsin has been found in Europe, Australia, New Zealand, and Asia (Hawkins *et al.*, 1997; Saker and Griffiths, 2001; Fastner *et al.*, 2003), but it should be considered a potential worldwide problem. Cylindrospermopsin and deoxycylindrospermopsin have been produced by *C. raciborskii* (Ohtani *et al.*, 1992), and 7-epicylindrospermopsin has been produced by *Aphanizomenon ovalisporum* (Banker *et al.*, 1997). After oral exposure, the LD<sub>50</sub> of cylindrospermopsin obtained with culture extracts of *C. raciborskii* ranged from 4.4 to 6.9 mg/kg in equivalent cylindrospermopsin (Seawright *et al.*, 1999; Shaw *et al.*, 2000).

Nodularins are cyclic pentapeptides that lead to severe hepatotoxicosis in the same way as microcystins (Harding *et al.*, 1995). In addition, nodularin is a more potent tumor promoter than microcystin (Sueoka *et al.*, 1997). The only cyanobacterium species known to

produce nodularin is *Nodularia spumigena*. This cyanobacterium can form extensive blooms in the Baltic Sea and in brackish waters in the summer (Francis, 1878; Sivonen *et al.*, 1989). The risk of nodularin intoxication is twofold because toxin exposure can occur not only through recreational or drinking water but also via contamination of seafood (Van Buynder *et al.*, 2001). In cases of acute hepatotoxicity, exposure to microcystins as well as nodularins must be considered.

$\beta$ -N-methylamino-L-alanine (BMAA), a neurotoxic amino acid, has been confirmed to be produced by a newly discovered cyanobacterial species associated with avian vacuolar myelinopathy (Bidigare *et al.*, 2009). The algal species is in the order Stigonematales and is found on the surface of *Hydrilla verticillata*, aquatic vegetation commonly found in wetlands in the southeastern United States (Wiley *et al.*, 2007). BMAA is a neurotoxic amino acid that has been associated with the pathogenesis of human amyotrophic lateral sclerosis–parkinsonism–dementia complex of Guam (Guam ALS-PD) and lathyrism. Birds with avian vacuolar myelinopathy develop ataxia, tilting, weakness, and death, and they have characteristic postmortem lesions of bilateral symmetrical vacuolation of the white matter of the brain and spinal cord (Thomas *et al.*, 1998). Characterization of BMAA provides a critical tool to study the impact of this cyanobacterial toxin on animals (and humans) and to develop control strategies.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

The frequency and extent of harmful cyanobacterial blooms appears to increase with the addition of nutrients to surface freshwater ecosystems throughout the world. As demonstrated in Table 72.1, numerous cyanobacterial species have the potential to produce a number of toxins with a range of target organs, but for each cyanobacterial species, it is very difficult to predict the nature and the level of the toxin production for a specific bloom. Although newer detection methods allow for better monitoring of potentially harmful blooms, there is still a need to apply these existing methods spatially and temporally and to develop lower cost, field-ready alternatives accessible to even remote areas. It is also important to develop more sophisticated methods that allow testing for a wider range of cyanotoxins and matrices in order to reliably confirm intoxications and improve overall risk assessment. Some recently developed methods have been useful in analyzing biological specimens in order to confirm a diagnosis of poisoning, but due to lack of availability and high cost, they are rarely pursued

in suspect cases. The lack of methods to confirm exposure is most likely responsible for the low number of reported cases in the veterinary literature during the past 20–30 years. The incorporation of new analytical methods into diagnosis should provide insight into the true frequency of cyanotoxin poisoning in animals. For example, in the United States, several dog intoxications due to anatoxin-a have recently been identified in different regions in relation with the development of an analytical method using high-performance liquid chromatograph–mass spectrometry analysis (Puschner *et al.*, 2008, 2010). In addition, information is needed on the efficacy of therapeutic measures. Similar to advisories for human populations, it is advisable to take preventative measures to avoid contact with surface waters containing visible blooms for all veterinary species, particularly when the water is utilized for a drinking source or for bathing.

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## Terrestrial zootoxins

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### INTRODUCTION

The Animal Kingdom is populated by a vast variety of creatures whose main focus in life is to live to see tomorrow. To this end, many animals have developed chemical means of defense and/or food procurement. Every phylum within the Animal Kingdom contains species that produce poisons or venoms. Poisons are compounds produced in nonspecialized tissues as secondary products of metabolism that accumulate in the host animal or that accumulate in predators following ingestion of prey. Poisonous animals, therefore, lack means of actively delivering these chemical compounds to others – exposure generally requires oral contact (rarely dermal) in order for another animal to become poisoned. In contrast, venoms are produced in specialized tissues or glands, and venomous animals have developed a variety of venom apparatuses (stingers, teeth, etc.) to deliver their venom to target animals – a process termed envenomation.

Most venoms and poisons are not composed of a single chemical substance but, rather, are mixtures of a variety of chemical compounds that often act synergistically to produce their toxic effect. Typical constituents include peptides, amines, serotonin, quinones, polypeptides, and enzymes (Russell, 2001). These compounds are collectively termed toxins (the science of the study of toxins is termed toxinology), and toxins produced by members of the Animal Kingdom are collectively termed zootoxins.

Not every exposure to a poisonous or venomous animal will necessarily result in a toxicosis (Meier, 1995). Variations in size, age, sex, season, toxin composition, and geographic location can result in zootoxins with relatively more or less toxicity. Similarly, the animal exposed to the zootoxin may be more or less susceptible

to toxicosis based on its age, weight, sex, and degree of exposure.

Compared to other means of injury or illness in animals, envenomation or poisoning from zootoxins are relatively rare causes for presentation of domestic animals to their veterinarians, due largely to the protection afforded by animal owners. Wildlife, on the other hand, probably face potential exposure to zootoxins on a nearly daily basis. Bites and stings from arthropods and snakes certainly can occur in any species, and the potential for oral exposure to animals such as poisonous toads, snakes, or insects will vary with the region and environment. Recent studies suggest that zootoxins can affect various vital organs, such as nervous, cardiovascular, and reproductive and developmental systems (Dorce *et al.*, 2009; Gwaltney-Brant, 2011; Troncone *et al.*, 2011).

In managing known or suspected zootoxicoses, veterinarians should be mindful to “treat the patient, not the poison.” Patient assessment and stabilization, along with supportive care, are critical in managing these patients. Although antivenins are available for treatment of some types of envenomation, financial and availability issues may limit their use in many instances.

### ARTHROPODA

#### ARANEAE: SPIDERS

##### Introduction

There are at least 30,000 species of spiders distributed throughout the world (Goddard, 2003). Spiders are



characterized by a two-segmented body: a head/thorax (prosoma or cephalothorax) and the abdomen (opisthosoma). They have eight segmented legs (Lucas and Meier, 1995b). Venom is stored in two glands located in the cephalothorax and empties through fangs (chelicerae) located at the rostral end of the prosoma (Lucas and Meier, 1995b). With the exception of spiders in the family Ulobiridae (found in Australia), all spiders are capable of inflicting an envenomating bite via fangs. Most spider envenomations, however, are likely to cause few signs other than local swelling and pain. Anaphylaxis and other allergic reactions to venom components are possible (Goddard, 2003). Although there is disagreement in the literature, it appears that fewer than 100 spider species can inflict a bite of medical significance (Lucas and Meier, 1995b).

## WIDOW SPIDERS (*LATRODECTUS* SPP.)

### Background

Spiders of this genus are found throughout the world. The spiders are identified by a red, yellow, or orange hourglass-shaped marking on the ventral abdomen of the female. Males and immature females are brown; the immature females do not have the hourglass marking. The fangs of the male are considered too short to inflict an envenomating bite to humans or other mammals (Lucas and Meier, 1995b). In the United States, there are five major species of widow spiders (Goddard, 2003):

1. *Latrodectus mactans* (black widow spider): These spiders are found throughout the United States but especially in the southern United States. Black widow spiders prefer dark places, especially under debris such as leaf litter or cardboard.
2. *Latrodectus hesperus* (western black widow spider): Common black widow of the western United States.
3. *Latrodectus variolus* (northern black widow spider): This species is common in the northern United States. The hourglass mark is not joined.
4. *Latrodectus bishopi* (red widow spider and red-legged widow spider): These spiders are found in palmetto fronds of the sandy, scrub-pine region of central and southern Florida. They are brightly colored.
5. *Latrodectus geometricus* (brown widow spider): The hourglass is orange in this species. These spiders are an introduced species to the United States found mainly in Florida.

Widow spiders are found mostly outside of living spaces, preferring dark secluded areas such as piles of debris and densely growing plants. Inside houses, they prefer dark, rarely disturbed spaces and will often settle

under appliances and cabinets. Adult spiders are most active in the warm months and will die during the colder months if not in a heated space. They build webs to ensnare prey. In general, widow spiders are shy and do not bite larger animals unless provoked (Goddard, 2003).

### Mechanism of action

Widow spider venom is a complex mixture of approximately six neuroactive proteins; there are also some proteolytic enzymes. The principal toxin for mammals is  $\alpha$ -latrotoxin, a polypeptide that causes a large release and then depletion of acetylcholine and norepinephrine at postganglionic sympathetic synapses (Lucas and Meier, 1995b).

### Toxicity

The venom of widow spiders is extremely potent. For instance, the LD<sub>50</sub> for whole venom of *L. mactans tridecimguttas* (European black widow) in guinea pigs is 0.0075 mg/kg and for mice is 0.9 mg/kg (White *et al.*, 1995).

The syndrome caused by widow venom is called latrodectism. Following a widow spider bite, the venom is taken up by lymphatics and then enters the bloodstream (White *et al.*, 1995). Clinically, there may be short-lived, localized pain at the bite site. Then, in approximately 30–120 min, myalgia and muscle cramps near the site of envenomation will begin. The pain begins to spread to the large muscles groups of the legs, thorax, back, and abdomen, peaking in approximately 2 or 3 h. Mild to moderate hypertension and tachycardia are common. In most cases, signs resolve in 48–72 h, but signs such as weakness, fatigue, and insomnia may persist for weeks to months (White *et al.*, 1995; Goddard, 2003).

Cats are very sensitive to the effects of widow venom. In one study, 20 of 22 cats died after widow spider bites, with an average survival time of 115 h. Paralysis occurs early in the course; severe pain is evidenced by howling and other vocalizations. Hypersalivation, restlessness, vomiting, and diarrhea were common. Muscle tremors, cramping, ataxia, and inability to stand may precede an atonic paralysis. Cheyne–Stokes respiratory pattern may develop prior to death (Peterson and McNalley, 2006a).

### Treatment

Treatment is largely symptomatic, with control of pain by opioids and use of muscle relaxants such as diazepam and methocarbamol to control the muscle rigidity. Calcium gluconate, which was once used for treatment

of widow envenomation, is no longer recommended because it was found to be less effective at controlling pain than opioids and muscle relaxants (Clarke *et al.*, 1992; Peterson and McNalley, 2006a). An equine-origin antivenin (Lycovac, available in the United States from Merck and Co.) has been used in humans. It is generally administered to high-risk patients (the very young or old) or in those whose signs are progressing despite other medical care. The antivenin is extremely effective; in one study, all 58 patients receiving the agent had resolution of signs within 30 min (Clarke *et al.*, 1992). In one case report (Twedt *et al.*, 1999), a cat with suspected lactrodectism was treated with antivenom approximately 26 h after signs began. The cat rapidly recovered neurologic function.

## RECLUSE OR VIOLIN SPIDERS (*LOXESCELES* SPP.)

### Background

Although there are at least 50 species of *Loxosceles*, few are capable of inflicting a medically significant envenomation known as loxoscelism. In the United States, where several *Loxosceles* species occur, the brown recluse spider (*L. reclusa*) is considered the only one of medical importance. *Loxosceles reclusa* is found principally in Missouri, Arkansas, Oklahoma, Kansas, and Tennessee, but its range extends from the southern Gulf states and southern Atlantic states through Indiana and Illinois. Other species of *Loxosceles* are found principally in the southwest United States and California. In South America, *L. laeta* and *L. gaucho* have been associated with severe loxoscelism in Brazil, Chile, Argentina, Peru, and Uruguay (White *et al.*, 1995). (*L. laeta* appears to have been introduced into the Los Angeles area (Goddard, 2003).) Although species of *Loxosceles* may occur in other regions of the world, significant morbidity and mortality have not been associated with these spiders (White *et al.*, 1995). Brown recluse spiders (*L. reclusa*) are nocturnal, nonaggressive spiders. They are recognizable by the “fiddle”-shaped marking on the dorsal surface of the cephalothorax.

### Mechanism of action

The venom of the recluse spider contains several necrotizing enzymes, including hyaluronidase, esterases, and alkaline phosphatases. Sphingomyelinase D, a phospholipase, appears to be the most important component; it binds to cell membranes and causes migration and activation of neutrophils in the area of the

envenomation. In addition, the venom inactivates serum hemolytic complement, leading to intravascular coagulation, occlusion of small capillaries, and tissue necrosis; systemic depletion of clotting factors (VII, IX, XI, and XII) can also occur (White *et al.*, 1995; Goddard, 2003; Peterson and McNalley, 2006b). Platelet activation can also be seen (Peterson and McNalley, 2006b). In the presence of calcium and C-reactive protein, sphingomyelinase D can cause hemolysis. Finally, lipases can cause free lipids in the blood that may act as inflammatory mediators and/or cause embolization (Peterson and McNalley, 2006b).

### Toxicity

In one study (Denny *et al.*, 1964), dogs injected intravenously with brown recluse venom developed thrombocytopenia, absence of reticulocytes, and evidence of hemolysis with decreased hematocrit. Necropsy showed widespread petechial and ecchymotic hemorrhage, dehydration, and hypocellular bone marrow with depression of the erythroid and platelet cell lines. Another study (Futrell *et al.*, 1979) of *in vitro* hemolysis found that human and pig erythrocytes were far more susceptible to lysis from brown recluse venom than those of dogs.

Initially, the bite may produce little pain or local reaction. Approximately 3–8 h after envenomation, the site becomes red, swollen, and tender; this is a so-called “bull’s-eye” lesion. A vesicle may form and be replaced by a black scab or eschar. Tissue around the bite may slough, leaving a 1- to 25-cm ulcer. Distribution may occur in areas dependent to the bite due to gravity. Healing is slow and may take months, often leaving a large scar (Goddard, 2003). Diagnosing a brown recluse bite may be difficult if the bite is not witnessed. In many cases, a brown recluse bite is “blamed” for necrotic lesions due to other causes (Mullen, 2002b). Systemic signs, although uncommon, can be seen with a brown recluse bite; they may develop 48–72 h after exposure. Hemolysis with anemia and hematuria, tachycardia, pyrexia, myalgia, vomiting, dyspnea, disseminated intravascular coagulation, and coma have been reported but are rare (Goddard, 2003).

### Treatment

For the necrotic lesion, local wound care including chemical debridement with Burrow’s solution (aluminum acetate) or hydrogen peroxide and bandaging should be performed. Pruritus may be controlled with diphenhydramine (2.2 mg/kg q8h). Antibiotics, especially if infection is evident, should be administered. Analgesics for

pain (NSAIDs for mild pain and opioids for severe pain) may be required. Surgical excision, used in the past, is no longer recommended (Peterson and McNalley, 2006b).

Dapsone (4,4'-diaminodiphenylsulfone) may be helpful in limiting the severity of the necrotic lesion because it inhibits neutrophil migration. In dogs, a dose of 1 mg/kg/day for 14 days has been used experimentally. Dapsone can cause methemoglobinemia as well as hemolysis in individuals with glucose-6-phosphate dehydrogenase deficiency (Peterson and McNalley, 2006b).

Antivenins are available for South American *Loxosceles* species. Experimental antivenins for *L. reclusa* have been developed. These have been shown to be effective if given within 4 h of envenomation. However, these antivenins are not currently commercially available (Mullen, 2002b).

## HOBO SPIDERS (*TEGENARIA AGRETIS*)

### Background

Hobo spiders are native to Europe but were introduced in the Pacific Northwest in the 1930s. They occur mainly in Washington, Oregon, and Idaho, but their range includes central Utah through western Canada to the Alaskan panhandle. In the 1980s, the appearance of necrotic spider bites in the Pacific Northwest was initially blamed on *L. reclusa*, but later the bites were correctly attributed to *T. agretis* (Goddard, 2003).

Hobo spiders are poor climbers and build a funnel web at ground level or in basements. Males will leave their web at night in search of females and may enter houses while doing so. Males are more venomous than females and are more likely to bite (Mullen, 2002a,b; Goddard, 2003).

### Toxicity

In most cases, the bite is initially painless. Within 30 min, a localized expanding area of erythema may occur; the lesion may eventually reach 15 cm in diameter. Then, in approximately 15–35 h, the area ruptures and there is a serous discharge from the wound. The wound may require 3 years to heal, especially if it is in fatty tissue (Goddard, 2003).

In humans, systemic signs may occur in approximately 45% of bites, and approximately one-third of these may require hospitalization. Signs may include headache (which can last for days), nausea, weakness, and vision changes. The signs may progress to vomiting (often intractable), watery diarrhea, and bone marrow

destruction resulting in anemia, pancytopenia, and thrombocytopenia. Fatalities are rare (Mullen, 2002b).

### Treatment

Treatment of the hobo spider bite is supportive and symptomatic.

## FUNNEL WEB SPIDERS (*ATRX* AND *HADRONYCHE* SPP.)

### Background

There are approximately 35 species of funnel web spiders. They are all found in Australia, principally distributed along the southeast coastal region. Although all may potentially be dangerous to mammals, the Sydney funnel web spider (*A. robustus*) is considered the most dangerous because this is the only species for which human fatalities have been recorded (White *et al.*, 1995). *Atrax robustus* is found within 160 km (100 miles) of the center of Sydney. They are large, aggressive black spiders (Sutherland and Tibballs, 2001).

### Mechanism of action

The toxic component of the funnel web spider's venom is the neurotoxin robustoxin, a 42-amino acid protein. The toxin binds to the presynaptic neuron and both inhibits central nervous system (CNS)-mediated neurotransmitter release and increases spontaneous release of neurotransmitters; the toxin affects both the autonomic nervous system and skeletal muscles. Other species of funnel web spiders contain structurally similar neurotoxins that are less potent than robustoxin (White *et al.*, 1995).

### Toxicity

There is a wide variation of reaction in different species of animals; the sex of the spider also affects toxicity of the bite. For instance, rats, rabbits, and cats are unaffected by the bite of a female spider, whereas 20% of mice and guinea pigs died after the bite of a female. When mice or guinea pigs are bitten by a male spider, most die. The male funnel web spider's venom appears to be six times more potent than that of the female based on minimum lethal dose determinations. However, in dogs and cats, male funnel web spider bites cause mild transient effects. Humans and other primates appear

to be extremely sensitive to the venom of this spider (Sutherland and Tibballs, 2001).

## TARANTULAS

Tarantulas are large, ground-dwelling spiders. During mating season, males wander in search of females and may be more aggressive at these times. In most cases, the bite of the tarantula causes little more than localized pain that develops slowly but usually resolves within 30 min. There are approximately 12 genera of tarantulas that may deliver a potentially life-threatening bite. These spiders occur in the tropical regions of South America, Africa, and Australia. Their venom contains a neurotoxin; there may also be a necrotoxin and hemolytic toxin. Following envenomation, muscle spasms, edema, hemoglobinuria, jaundice, and circulatory shock may develop (Mullen, 2002b).

Tarantulas of the family Theraphosidae (bird-eating or whistling spiders) are often kept as pets in Australia and Asia. In one Australian study (Isbister *et al.*, 2003), the bite of these spiders was found to cause only local effects in humans. However, seven dogs bitten by spiders of the genera *Phlogiellus* and *Selenocosmia* died rapidly, often within 2 h of the envenomation. (In two cases, the dogs and their owners were bitten by the same spider.) The spiders are also considered highly poisonous for cats. The exact cause of death was not discussed, but based on experimental data, apnea and cardiac arrhythmias may occur.

Species of tarantula living in the United States are not capable of delivering serious envenomation. However, many of these species have urticating hairs on their abdomen that possess spines and barbs that can penetrate skin. When threatened, these tarantulas can stroke their abdomen and flick the urticating hairs at their attacker. The hairs can cause severe inflammation of the skin, eyes, mouth, and respiratory tract. The effect is purely mechanical because there is no toxin associated with the hairs. Urticaria, edema, and vasodilation may occur in the skin. In the eyes, swelling of the lids and corneal abrasions are common (Mullen, 2002b). Dogs or cats attempting to ingest tarantulas may gag or vomit.

## SCORPIONES: SCORPIONS

### Background

Scorpions are arachnids with two body divisions: the combined head and thorax, referred to as the prosoma or cephalothorax, and the abdomen or opisthosoma.

**TABLE 73.1** Scorpions of medical importance: species and location

Location	Species
India	<i>Buthotus tamulus</i>
Mexico and Central America	<i>Centruroides</i> spp.
Middle East	<i>Androctonus</i> spp. <i>Buthus</i> spp. <i>Hemiscorpion lepturus</i> <i>Leiurus quinquestriatus</i> <i>Mesobuthus gibbosus</i>
South Africa	<i>Parabuthus</i> spp. <i>Buthus minax</i>
South America	<i>Tityus</i> spp. <i>Centruroides gracilis</i>
United States	<i>Centruroides sculpturatus</i>
West Indies (Trinidad)	<i>Tityus trinitatis</i>

Adapted from Lucas and Meier (1995a) and Keegan (1980).

They possess large paired claws or pincers known as pedipalps and four pairs of legs. At the caudal portion of the abdomen, scorpions have a tail that terminates in the telson – a barbed appendage that houses two venom glands that exit via a stinger at the end of the telson. Scorpions are nocturnal hunters; they may spend the day in burrows or hiding under rocks or vegetation. They may also hide in blankets, shoes, and clothing; this a common way for humans to be exposed to them (Keegan, 1980).

There are approximately 1400 species of scorpions occurring on all continents except Antarctica (Goddard, 2003). Most occur in tropical or temperate regions (Lucas and Meier, 1995a). Table 73.1 lists locations and species of scorpions capable of inflicting life-threatening envenomations in humans. The main genera of scorpions found in North America are the bark scorpions (*Centruroides* spp.). They are found primarily in Central America, Mexico, and the southern through southwest United States (Mullen and Stockwell, 2002). In the United States, only *Centruroides exilicauda* (Arizona bark scorpion) is considered capable of inflicting a life-threatening sting (Goddard, 2003).

### Mechanism of action

All scorpions can deliver an envenomating sting (Keegan, 1980). Scorpion venom components vary greatly between genera and may even differ based on geographic location within species (Mullen and Stockwell, 2002). The venom consists of a mixture of low-molecular-weight polypeptides. At least two potent neurotoxins have been identified:  $\alpha$ -scorpion toxin found in *Androctonus*, *Leiurus*, and *Buthus* spp. and  $\beta$ -scorpion toxin found in *Centruroides* spp. Both toxins can be found in the venom of *Tityus* spp. These



venoms block the voltage-sensitive sodium and potassium channels in nerves (Mullen and Stockwell, 2002).

## Toxicity

Scorpion stings cause instant, sharp pain at the site of envenomation. Some stings will cause localized pain that resolves over hours. Localized edema and pruritus are common. Regional lymph nodes may enlarge, and there may be an allergic reaction characterized by swelling of the eyelids, tongue, and vomiting. Sloughing of the skin at the site of envenomation can also occur (Mullen and Stockwell, 2002). Signs usually resolve within 24h (Keegan, 1980). Systemically, signs can vary but generally include numbness of face, myalgia, tachycardia or bradycardia, respiratory depression, and seizures; however, there is a lack of evidence that scorpion envenomation in dogs and cats is a serious concern (Mullen and Stockwell, 2002).

## Treatment

In most cases, the treatment of scorpion stings consists of analgesics and local wound care. Systemic signs are treated symptomatically with control of hypertension, heart rate changes, and neurologic signs. Some antivenoms in the United States are produced locally; however, because these are not approved by the U.S. Food and Drug Administration, they cannot legally be transported to other states (Goddard, 2003). In addition, their use in veterinary patients is considered controversial (Dalefield and Oehme, 2006).

# IXODIDA: TICKS

## Background

Ticks are well known as being vectors for a large number of human and animal diseases, with the first reports of tick paralysis originating in Australia in 1890 and British Columbia in 1912 (Sonenshine *et al.*, 2002). Worldwide, 43 species of ticks from nine different genera have been associated with tick paralysis: *Amblyomma*, *Argas*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Ornithodoros*, *Otobius*, and *Rhipicephalus* (Dipeolu, 1976; Fowler, 1993). Tick paralysis has been reported in North America, Europe, Africa, Australia, and the former Soviet Union.

## Mechanism of action

The exact mechanism(s) of action of tick toxins is not well known, but in most tick species it is suspected that the toxin interferes with the synthesis and/or release of

acetylcholine at the neuromuscular junctions, resulting in lower motor neuron paresis and paralysis very similar to that produced by botulinum toxin (Fowler, 1993; Grattan-Smith *et al.*, 1997). The Australian tick, *Ixodes holocyclus*, toxin may differ because it appears to have more of an effect on central nerve centers rather than on peripheral (Fowler, 1993).

## Toxicity

Tick paralysis has been reported in a large variety of animal species, including dogs, cats, cattle, sheep, goats, llamas, poultry, wild antelope, bison, foxes, wolves, mice, ground hogs, black-tailed deer, and several species of wild birds (Daly *et al.*, 1992; Fowler, 1993; Beyer and Grossman, 1997; Sonenshine *et al.*, 2002). In North America, most cases of tick paralysis in livestock occur in the Pacific Northwest due to *Dermacentor andersoni*, whereas most cases in dogs are due to *D. variabilis* (Fowler, 1993; Sonenshine *et al.*, 2002). *Dermacentor occidentalis* occasionally causes tick paralysis in cattle, ponies, and deer but not dogs (Sonenshine *et al.*, 2002). In the southeastern United States, *I. brunneus* has been associated with tick paralysis in wild passerine birds (Luttrell *et al.*, 1996). In Australia, tick paralysis is caused by *I. holocyclus*, whereas *I. rubicundus* and *Rhipicephalus evertsi* are the primary species responsible for tick paralysis in Africa (Sonenshine *et al.*, 2002). Tick paralysis in Europe and Asia has been reported due to bites from *Hyalomma punctata* and *I. ricinus*.

Tick paralysis has occurred following the bite of as few as one tick, and heavily infested animals may succumb quickly (Fowler, 1993). Clinical signs include an ascending ataxia that progresses to paresis and flaccid paralysis. Early in the intoxication, animals remain bright, alert, and able to eat and drink if properly supported. Eventually, paralysis of the respiratory muscles leads to respiratory failure and death. Paralysis produced by *I. holocyclus*, the Australian tick, generally occurs more rapidly and tends to persist following removal of the tick.

## Treatment

The main goal of treatment is to remove the ticks and provide supportive care (especially respiratory support) until recovery occurs. Recovery can occur quite rapidly following complete removal of the ticks, or it may take a few days (Fowler, 1993). The use of topical insecticides may aid in the removal of ticks and can be especially helpful in cases in which numerous ticks are embedded. Heavily coated animals may need to be shaved to ensure that all embedded ticks are found and removed. Removal of embedded ticks should be performed carefully to avoid expressing additional toxin into the wound or leaving the head embedded in the skin. Forceps may be used to grasp

the tick as close to the skin as possible, and gentle traction should be used to remove the tick. In most cases in which ticks are removed before bulbar paralysis has occurred, the prognosis for full recovery is very good. A short-term immunity develops following recovery from tick paralysis. In Australia, a polyclonal dog antiserum has been developed to treat tick paralysis, but it is only effective in the early stages of paralysis (Masina and Broady, 1999).

## MYRIAPODA: CENTIPEDES AND MILLIPEDES

Centipedes and millipedes are distantly related to lobsters, crayfish, and shrimp. These arthropods are widely distributed throughout the world, and they are characterized by a long, flat, multisegmented body with one (centipede) or two (millipede) legs emerging from each body segment. Although most centipedes and millipedes are less than 1.5 in. long, centipedes from the genus *Scolopendra* may grow to be 4–6 in. long. All centipedes have a pair of modified front legs (forcipules) that serve as fangs and that are connected to venomous glands directly under the head (Norris, 2004). Larger centipedes have the ability to inflict painful bites resulting in local swelling, erythema, and lymphangitis (Stewart, 1985). In addition, the legs of *Scolopendra* spp. are tipped with sharp claws that are capable of penetrating skin, and toxin produced at the attachment point of each leg may drop into these wounds, causing inflammation and irritation.

Centipede venom has been poorly studied, but components identified in various centipedes include serotonin, a cytotoxin, a hemolysin, and a vesicating agent (Deng *et al.*, 1997; Norris, 2004). In most cases, systemic toxicosis is not expected, although local necrosis may occur (Russell, 1996); a single case report exists of a human fatality from the bite of *Scolopendra subspinipes* (Norris, 2004). Signs generally subside within 48h and rarely require more than symptomatic care (e.g., analgesics). Millipedes do not bite but can emit irritating and foul-smelling secretions from repugnatorial glands; some species can spray these fluids over distances of several inches. These secretions are irritating to mucosal surfaces, particularly the eyes, and corticosteroids have been recommended to decrease the inflammatory response (Russell, 1996).

## INSECTA: INSECTS

### Introduction

Insect and insect-related problems are common in domestic and wild animals. Insects such as lice, fleas, deerflies, horseflies, sand flies, mosquitoes, blackflies,

and biting midges may cause severe annoyance to animals because of biting behavior. Members of several groups of insects can inject venom when they bite or sting, most notably bees, wasps, and ants. Bites or stings from insects such as lice, fleas, bees, ants, wasps, mosquitoes, and chiggers may cause direct effects from the venoms or may result in allergic host reactions resulting from overresponsive host immune systems. Contact allergies may occur when certain beetles or caterpillars touch the skin. Other insects, such as blister beetles and certain caterpillars, produce toxins that can cause adverse reactions when they are touched or ingested (Durden and Mullen, 2002). Respiratory allergies can result from inhaling allergic air-borne particles from cockroaches, fleas, or other arthropods (Durden and Mullen, 2002). Some insects invade the body tissues of their host. Various degrees of invasion may occur, ranging from subcutaneous infestations to invasion of organs such as the lungs and intestine (Durden and Mullen, 2002). Table 73.2 provides a list of nine orders of insects that are of particular interest to veterinary entomologists.

## COLEOPTERA: MELOIDAE (BLISTER BEETLES)

### Background

More than 300,000 species of beetles have been described, representing 30–40% of all known insects. Approximately 25,000 species of beetles occur in the United States and Canada (Krinsky, 2002). Fewer than 100 species of beetles worldwide are known to be of public health or veterinary importance. Most of these are in the suborder Polyphaga. The species that have the greatest impact on the health of human and domestic animals are in the following families: Melidae (blister beetles), Oedemeridae (false blister beetles), Staphylinidae (rove beetles), Tenebrionidae (darkling beetles), Dermestidae (larder beetles), and Scarabaeidae (scarab or dung beetles) (Krinsky, 2002).

Most blister beetles are in the family Melidae, although some are in the Staphylinidae family. Blister beetles are elongate, soft-bodied specimens in which the pronotum (section between head and wings) is narrower than the head or wings. Two of the common blister beetle species are potato beetles, one with orange and black longitudinal stripes and one black with gray wing margins (Goddard, 2003). Members of the genus *Mele* are called oil beetles because they exude an oily substance from their legs when disturbed. Oil beetles are approximately 20–25mm long and black with no hind wings, giving the appearance that their wings are too short (Goddard, 2003).

TABLE 73.2 Insects of veterinary importance

Order	Common names	Clinical significance
Coleoptera	Beetles	Toxic beetles (e.g., blister beetle); vectors for pathogenic organisms; intermediate host for nematodes, flukes, cestodes, acanthocephalans
Dictyoptera	Cockroaches	Intermediate hosts for nematodes, flukes, cestodes, acanthocephalans; vectors for pathogenic organisms
Diptera	Flies, mosquitoes	Vectors for pathogenic organisms; myiasis; decreased production due to annoyance or anemia from biting/sucking insects
Hemiptera	True bugs	Transmission of infectious agents (e.g., trypanosomiasis); decreased production due to anemia from blood-sucking bugs
Hymenoptera	Wasps, hornets, ants, bees	Envenomation, allergy from bites or stings; poisonous insects (e.g., sawfly larvae)
Lepidoptera	Moths, butterflies	Urticaria, irritation, ulceration from urticating hairs; poisonous insects (e.g., monarch butterfly)
Phasmida	Stick insects, leaf insects	Irritation from secretions of walking sticks
Phthiraptera	Lice	Decreased production due to annoyance or anemia; vector for pathogenic organisms
Siphonaptera	Fleas	Decreased production due to annoyance or anemia; vector for pathogenic organisms

Species that pose problems in the United States include the striped blister beetle (*Epicauta vittata*) (Figure 73.1), the black blister beetle (*E. pennsylvanica*), the margined blister beetle (*E. pestifera*), and the three-striped blister beetle (*E. lemniscata*), as well as *E. fabricii*, *E. occidentalis*, and *E. temexa*. Of these, the most common species associated with toxicosis are *E. occidentalis* and *E. temexa* (Stair and Plumlee, 2004).

Mechanism of action

The toxic principle in melioid beetles, cantharidin, has the formula  $C_{10}H_{12}O_4$  and is a bicyclic terpenoid (Goddard, 2003; Stair and Plumlee, 2004). The concentration of cantharidin present in beetles varies from 1 to 5% of the dry weight of the beetle. Males always have a higher concentration because the male produces the cantharidin and transfers a quantity to the female during copulation (Krinsky, 2002). Cantharidin is present in the hemolymph as well as in the clear, yellow secretion that is exuded at the joints of the legs of these beetles by reflex bleeding (Figure 73.2).

The mechanism of action of cantharidin may involve interference with enzyme systems responsible for active transport across mitochondrial membranes, resulting in membrane disruption and permeability changes. Mitochondrial damage results in cellular disruption, acantholysis, and vesicle formation. Cantharidin has also been shown to inhibit protein phosphatase 2A, resulting in disruption of signal transduction and cell metabolism (Stair and Plumlee, 2004).

Purified, crystalline cantharidin is a potent vesicating agent that is readily absorbed through the gastrointestinal mucosa and, to a lesser degree, the skin. It is eliminated unchanged through the kidneys (Krinsky, 2002).



FIGURE 73.1 An adult striped blister beetle (*E. vittata*). (Photo courtesy of James E. Appleby, University of Illinois.)



FIGURE 73.2 An adult blister beetle (*Epicauta*), with hemolymph droplets containing cantharidin. (Photo courtesy of Dr. Maria Eisner, Cornell University.)



## Toxicity

Adult blister beetles feed on flowering foliage, especially blooming alfalfa (*Medicago sativa*). Blister beetles in alfalfa fields contain enough cantharidin to provide lethal doses to livestock that feed on forage when it is used as hay. Modern methods of hay harvesting, especially crimping, decrease the opportunity of blister beetles to vacate the foliage before it is incorporated into hay, thereby increasing the likelihood of blister beetles being trapped in the hay (Stair and Plumlee, 2004). Cantharidin released from crushed adult blister beetles may contaminate hay without visual evidence of insect parts. Cantharidin is very stable and may persist in hay for long periods of time. Because in the United States alfalfa is most widely grown in the South, cantharidin toxicosis is most commonly found in the southern states; however, due to interstate transport of alfalfa hay throughout the country, cantharidin toxicosis could potentially occur in any state.

Horses are the species in which cantharidin toxicosis is most commonly reported. However, the following hosts have also been poisoned in natural or experimental situations: cattle, sheep, goats, rabbits, rats, hedgehogs, dogs, and emus. Poisoning in human beings has been noted for decades and is usually the result of either improper medicinal use of cantharides or malicious poisoning (Krinsky, 2002). Horses are considered to be particularly susceptible to the effects of cantharidin (Stair and Plumlee, 2004). The estimated lethal dosage of cantharidin for the horse is approximately 0.5–1 mg/kg, and as little as 4 g of dried beetles may be lethal to a horse (Krinsky, 2002). The oral experimental lethal dosage of crystalline cantharidin for dogs and cats was 1.0–1.5 mg/kg, and it was 20 mg/kg for rabbits. It is estimated that the lethal dosage for a human is less than 1.0 mg/kg.

Cantharidin produces an intense, direct irritant effect on the skin and mucous membranes of the esophagus, stomach, and intestines. Once absorbed, the toxin may affect many different organs. Excretion is via the kidneys, resulting in transfer of the irritant effect to the urinary tract, particularly the bladder and urethra (Krinsky, 2002).

Clinical signs vary with the dose ingested. Massive doses may cause shock and death within 4 h (Krinsky, 2002). Smaller doses may cause gastroenteritis, nephrosis, cystitis, and/or urethritis; thus, signs may include anorexia, soft feces, mucoid to bloody feces, intestinal atony, colic, dysuria (frequent, painful urination, or oliguria to anuria), and hematuria. The body temperature may elevate to 106°F (41.1°C). Other signs observed include depression, weakness, muscle rigidity, collapse, prostration, dehydration, and sweating (Krinsky, 2002). Animals frequently become dyspneic, and rales may be detected on auscultation due to pulmonary edema. Myocarditis may initiate cardiovascular signs including tachycardia, congested mucous membranes,

and decreased capillary refill time. Synchronous diaphragmatic flutter and muscle fasciculations have been reported in horses and are thought to be a result of hypocalcemia (Stair and Plumlee, 2004). Ulceration of the oral mucosa membranes may be observed, and animals may be seen dipping their muzzles into water without drinking (Krinsky, 2002). Diarrhea may be observed in animals that live for a few days. The course of the disease may be as short as 4 h, with massive dose ingestion, to 5 days in lethal poisoning. In horses, the mortality rate is approximately 50%, with horses surviving more than 1 week having a favorable prognosis (Krinsky, 2002).

In both field cases and experimental cantharidin poisoning, there are elevations in serum protein and packed cell volume caused by dehydration and shock. The damaged gastrointestinal mucosa allows rapid invasion of enteric bacteria, resulting in bacteremia and leukocytosis. There may be mild elevation in serum urea nitrogen. Profound hypocalcemia (5.9 mg/dL; normal, 12.8 ± 1.2 mg/dL) and hypomagnesemia (0.7–1.8 mg/dL; normal, 2.5 ± 0.3 mg/dL) have been reported. Specific gravity of urine is low in the early stages of the disease, and erythrocytes are usually present in the urine, yielding a positive occult blood reaction.

Gross lesions may be minimal with massive dose ingestion. In more protracted cases, oral ulcers, vesication and desquamation of patches of the distal esophagus, erosions and ulceration of the gastrointestinal tract, mucus in the renal pelvis, and renal cortical hemorrhages may be seen. Hyperemia and hemorrhages are seen in the urethra and bladder mucosa. Ventricular myocarditis, pulmonary edema, petechial hemorrhages of serosal surfaces, hepatomegaly, and splenomegaly may also be present.

The initial microscopic lesion is acantholysis of mucosa of the gastrointestinal tract, epithelium of the urinary tract, and endothelium of vessels (Krinsky, 2002). Other microscopic lesions include myocarditis, renal tubular nephrosis, and degenerative changes in the kidneys and digestive tract.

Cantharidin may be detected in urine, tissue (kidney and blood), gastrointestinal contents, and the dried beetles themselves by high-pressure liquid chromatography or gas chromatography–mass spectrometry. Cantharidin is excreted rapidly and may not be present in detectable amounts after 4 or 5 days following ingestion (Krinsky, 2002).

## Treatment

There is no specific treatment. The administration of either activated charcoal or mineral oil (but not together) via a gastric tube may aid animals that have consumed a small dose or are in the early stages of poisoning.



General supportive therapy should include correction of fluid loss and electrolyte imbalances, particularly hypocalcemia and hypomagnesemia (Krinsky, 2002).

Broad-spectrum antimicrobial therapy may be necessary to counter secondary bacterial invasion from the gastrointestinal tract. Aminoglycoside antibiotics and others that are potentially nephrotoxic or that are excreted via the kidney should be avoided because nephrosis is a potential effect of cantharidin.

## COLEOPTERA: FIREFLIES

### Background

Fireflies of the genus *Photinus* have been reported to cause death when fed to captive reptiles and amphibians (Knight *et al.*, 1999).

### Mechanism of action

Lucibufagins, steroidal pyrones structurally related to cardiotoxic bufodienolides of toads, have been isolated from the bodies of *Photinus*. It is suspected that lucibufagins have cardiotoxic effects similar to those of bufodienolides – that is, they inhibit sodium–potassium ATPase activity in the myocardial cell membrane in a mechanism similar to digitalis (Brubacher *et al.*, 1999).

### Toxicity

It is likely that the lucibufagins protect the fireflies from predation from birds and spiders (Knight *et al.*, 1999). Reported cases of firefly toxicosis have occurred when captive reptiles and amphibians were fed the fireflies by their owners. As few as one firefly has caused death in lizards. Toxicosis has been reported in bearded dragons (*Pogona* spp.), an African chameleon (*Chamaleleo* spp.), White's tree frogs (*Litoria caerulea*), and a *Lacerta derjugini* lizard.

Within 5–90 min of being fed fireflies, affected lizards displayed head shaking, oral gaping, dyspnea, skin color change to black, and regurgitation, with death occurring as rapidly as 15 min post-ingestion (Knight *et al.*, 1999). No gross lesions were reported in animals presented for postmortem.

### Treatment

Because of the rapid onset of signs and rapid progression to death, no veterinary care could be instituted in the reported cases (Knight *et al.*, 1999). Treatment for affected

animals would include management of any cardiac arrhythmias and general supportive care. Because the lucibufagins are similar in structure to bufodienolides, in theory one would expect that digoxin-specific Fab fragments (e.g., Digibind) might be of benefit in treating animals with firefly toxicosis (Brubacher *et al.*, 1999). However, given the rapid development and progression of clinical signs following ingestion of fireflies, the prognosis in most cases will be poor.

## COLEOPTERA: OTHER TOXIC BEETLES

Ingestion of rove beetles, *Paederus fuscipes* (Staphylinidae), has resulted in toxicosis in horses and cattle in Southeast Asia (Krinsky, 2002). Pederin, a toxin more potent than *Latrodectus* spider venom, can cause severe vesicular damage to the mucosa of the alimentary tract. Ingestion of the rose chafer (*Macrodactylus subspinosus*), a member of the Scarabaeidae, has been associated with deaths in chickens, ducklings, goslings, and young turkeys in North America, although modern confinement poultry production operations have largely reduced the risk of toxicosis.

In Michigan, a dog that ingested large numbers of Asian ladybeetles (*Harmonia axyridis*) developed severe shock, cyanosis, rapid pulses, and lateral recumbency (Hoenerhoff *et al.*, 2002). The dog subsequently died, and on postmortem large numbers of partially digested and intact Asian ladybeetles were detected in the small and large intestines associated with severe hemorrhage and thickening and ulceration of the jejunum and ascending colon. There was a pungent odor to the gastrointestinal contents that mirrored the foul-smelling fluid that Asian ladybeetles can produce when disturbed. Asian ladybeetles were introduced as a biologic control agent and are spreading rapidly throughout the United States.

## HYMENOPTERA: BEES, WASPS, AND HORNETS

### Background

Bee and wasp stings caused approximately 23 human deaths per year in the United States during the 1950s (Akre and Reed, 2002). During that same period, approximately 13 individuals per year died of snakebite. No statistics are available for fatal bee or wasp stings in animals. More than 20,000 species of bees are distributed throughout the world (Fowler, 1993). The honeybee, *Apis mellifera*, is one of two domesticated insect species (the other is the silk worm, *Bombyx mori*). Native honeybees in tropical Southeast Asia

were the source of the domestic honeybee. When early American colonists imported European strains of the honeybee to the Virginia Colonies, the bees became known as European honeybees. Africanized honeybees (*A. mellifera scutellata*) are a subspecies of the common honeybee native to Africa that has spread from South and Central America into south Texas, New Mexico, Arizona, Nevada, California, and Puerto Rico (Akre and Reed, 2002).

### Mechanism of action

There are numerous variations in the venom apparatus of members of Hymenoptera (Fowler, 1993). The stinger is a modification of the ovipositor apparatus and is found only in female bees and wasps. Venom secreted from specialized cells in the acid glands is transported to the venom sac reservoir via small tubules. One-way valves in the bulb of the venom apparatus control the flow of venom during envenomation. At the time of venom injection, the alkaline gland contributes a secretion that enhances the toxicity of the venom. The stinger of the honeybee is covered with retrograde barbs that cause the stinger to remain impaled in thick-skinned victims. When this occurs and the bee attempts to withdraw, the entire stinger apparatus is pulled from the bee, resulting in death of the honeybee.

Honeybee venoms are complex mixtures of proteins, peptides, and small organic molecules (Akre and Reed, 2002). Phospholipases and hyaluronidases present in the venom account for the majority of allergic responses to bee venoms in humans and likely other animals as well. Phospholipase A<sub>2</sub> is one of the most lethal peptides in honeybee venom (Schmidt, 1995). Mellitin is a membrane disruptive compound that increases the susceptibility of cell membranes to the damage caused by phospholipases within the venom (Akre and Reed, 2002). Mellitin can also cause pain, trigger hemolysis, increase capillary blood flow, increase cell permeability, and enhance spread of venom constituents within tissue. Mellitin, in combination with phospholipase and a mast cell degranulating peptide, triggers the release of histamine and serotonin. In mice, mellitin was found to be the primary lethal component of honeybee venom (Schmidt, 1995). Apamin is a neurotoxin that blocks calcium-activated potassium channels and has been associated with transient peripheral nerve effects in humans after bee stings (Saravanan *et al.*, 2004). In cats, bee venom can cause contraction of bronchiolar muscles.

Like honeybees, vespid wasps (including yellow jackets and hornets) produce venoms containing peptides, enzymes, and amines designed to trigger pain (Akre and Reed, 2002). The primary pain-inducing substances are kinins; however, other compounds present in vespid venom, such as serotonin, histamine, tyramine,

catecholamines, and acetylcholine, can contribute to the pain as well as local vasoactivity. Several of the constituents of vespid venom can act as allergens and trigger allergic reactions. Some vespid venoms contain neurotoxins or alarm pheromones that alert the swarm to an intruder.

### Toxicity

Honeybees can inflict only a single sting, but an animal attacked by a swarm or hive of bees may sustain multiple stings and the cumulative envenomation may be lethal. The Africanized honeybee (*A. mellifera scutellata*) presents a special case. Although its venom is no more toxic than that of the domestic honeybee, the aggressive behavior of the African honeybee increases the likelihood of multiple stings occurring by swarms of these bees (Akre and Reed, 2002). Wasps and hornets either lack a barbed stinger or the barbs are small and do not prevent withdrawal of the stinger. Therefore, a single wasp may inflict multiple stings. Also, because many wasps are highly social, multiple stings are commonplace.

A sting by a single bee or vespid rarely causes more than a transient, painful prick in animals, in contrast to humans, where sensitive individuals may die peracutely from a single sting. However, death following a single bee sting has been reported in a dog (Fowler, 1993). Deaths following attacks on livestock by Africanized bees have been reported, although the majority of interactions between livestock and bees or wasps result in only local reactions. Yellow jacket envenomation leading to skin lesions and death has been reported in a group of pigs (Fowler, 1993).

Local reactions to single bee, hornet, or wasp stings consist of swollen, edematous, and erythematous plaques at the site of the sting (Fowler, 1993). Most small animal patients present with facial, periorbital, and/or aural edema. Honeybee stingers will occasionally be located embedded in the area, and a small abscess may form at the site. In the Middle East, the German wasp, *Vespula germanica*, has been observed to injure the teats and udders of dairy cattle, causing lesions that can lead to mastitis. Apparently, the wasps served as a vector in spreading *Streptococcus dysgalactiae* infection in the herds (Yeruham *et al.*, 1998).

Multiple stings may produce numerous wheals, urticaria, and in severe cases may cause severe systemic responses as direct toxic effects of the venom. Systemic effects reported in dogs following multiple stings from bees or vespids include prostration, convulsions, CNS depression, shock, hyperthermia, bloody diarrhea, bloody vomiting, leukocytosis, intravascular hemolysis, disseminated intravascular coagulopathy, and elevations of blood urea nitrogen and alanine transaminase, suggesting renal and hepatic involvement (Wysoke *et al.*, 1990; Cowell *et al.*, 1991; Fowler, 1993). Hepatic injury in

cats has also been reported following exposure to hornet venom. Acute lung injury similar to the human acute respiratory distress syndrome developed in a dog following envenomation by more than 100 bees (Walker *et al.*, 2005).

The classic anaphylactoid response to bee and vespid venoms that is not uncommon in humans has not been documented in livestock, but anaphylaxis in dogs due to bee stings has been reported (Akre and Reed, 2002). Immune-mediated hemolytic anemia secondary to bee envenomation developed in two dogs (Noble and Armstrong, 1999). Clinical signs included lethargy, hematuria, ataxia, and seizures, and one dog died. Clinicopathologic data included nonregenerative anemia, spherocytosis, positive results for Coomb's test, and occult hematuria. Treatment included oral administration of corticosteroids at immunosuppressive dosages and supportive care. The surviving dog initially responded to corticosteroids, but hemolysis recurred as the dosage was tapered. Hemolysis resolved with prolonged administration of corticosteroids.

## Treatment

Ideally, when an insect has stung an animal, identification of the insect should be attempted. Most often, stings occur in areas that are free of hair or have short hair. For bee stings, the site should be examined to determine if any part of the stinger remains. Retained stingers should be scraped away from the injection site; grasping the stinger with forceps may result in more venom being expressed into the injection site. Cold compresses may be used as first aid to relieve pain and swelling. Antihistamines and corticosteroids are thought to have questionable value once lesions have developed, but they are not contraindicated (Fowler, 1993). Patients should be monitored for development of systemic or anaphylactic reactions, which need to be treated promptly and aggressively. Epinephrine may be used in cases in which anaphylaxis is suspected. In cases of true systemic toxicosis, aggressive intravenous fluid therapy is recommended using balanced fluid solutions. Other treatments that have been recommended include corticosteroids, antihistamines, oxygen for dyspnea, and diazepam as needed for convulsions (Fowler, 1993). Renal and hepatic function should be monitored in animals that develop systemic reactions. In most cases, exposure to single stings from bees and vespids is rarely fatal.

## HYMENOPTERA: ANTS

### Background

There are more than 10,000 species of ants, some of which bite, some of which sting, and others that both bite and sting. Some ants that lack a sting have the

ability to spray formic acid, which can cause local irritation to the victim if it gets in the eyes or wounds produced by the ant's mandibles.

Multiple stings from venomous ants have resulted in the death of pets, livestock, and wild animals in North, Central, and South America (Fowler, 1993). The red imported fire ant, *Solenopsis invicta*, is the most important species in terms of envenomation of animals. This ant was native to Brazil but entered the United States in the 1940s, where it has become a serious pest in the southeast as far west as Texas.

### Mechanism of action

Ant venoms, like bee venoms, are complex mixtures of compounds that can induce a variety of adverse effects in animals. Fire ant venoms consist largely of alkaloids, with less than 1% proteinaceous component (Akre and Reed, 2002). The alkaloids consist of solenopsins (methyl-*n*-alkylpiperidines) and a piperidine that cause dermal necrosis when injected in skin. These alkaloids have cytotoxic, hemolytic, fungicidal, insecticidal, and bactericidal properties.

### Toxicity

Animals most likely to be severely affected by fire ants tend to be those that are unable to move away from a colony, including those that are neonatal, juvenile, or disabled. Attacks of fire ants resulting in deaths of newborn game animals such as rabbits and deer have been reported (Akre and Reed, 2002). Nesting bird chicks and newly hatched quail and poultry have been killed (and often eaten) by fire ants (Fowler, 1993).

Clinical effects of fire ant envenomation include intense pain at the site of the sting. In humans, within minutes of a sting there is formation of an urticarial wheal, which progresses to a vesicle and then a pustule within 24 h (Fowler, 1993). Within a few days, the pustule will rupture, resulting in a crusting lesion. Dogs do not appear to develop the pustules described in humans; instead, they develop erythematous pruritic papules that generally resolve within 24 h (Rakich *et al.*, 1993). There are no reports of anaphylaxis in animals secondary to fire ant stings (Akre and Reed, 2002). Multiple stings may result in systemic signs similar to those of multiple bee or wasp stings.

### Treatment

Single bites from fire ants generally require no treatment. Multiple envenomations resulting in severe systemic

reactions or anaphylaxis should be managed similarly to systemic reactions to bee stings (i.e., fluids, corticosteroids, and supportive care).

## HYMENOPTERA: SAWFLIES

### Background

Sawflies are primitive hymenopterans that belong to the suborder Symphyta and are widely distributed throughout the world. Sawfly poisoning has been reported in livestock in Denmark by *Arge pullata*, in Australia by *Lophotoma* spp., and in Uruguay by *Perreyia flavipes* (Fowler, 1993; Thamsborg *et al.*, 1996; Dutra *et al.*, 1997).

### Mechanism of action

Sawfly larvae harbor D-amino acid-containing peptides that are considered the toxic principle in sawfly larvae poisoning, which results in hepatic, and occasionally renal, degeneration and also necrosis (Oelrichs *et al.*, 1977). Lophyrotomin, an octapeptide structurally similar to cyclic peptide hepatotoxins from *Amanita* mushrooms (Thamsborg *et al.*, 1996), is the major toxin found in the Australian and Danish sawfly larvae (Oelrichs *et al.*, 1999). The bile acid transport system is important in the hepatocellular uptake of lophyrotomin, which causes a periportal hemorrhagic necrosis of the liver (Oelrichs *et al.*, 2001; Chong *et al.*, 2002). Pergidin, a heptapeptide containing a phosphoserine residue, is the major toxin found in the South American sawfly. In contrast to lophyrotomin, pergidin causes a periacinar coagulative necrosis in the liver (Oelrichs *et al.*, 2001).

### Toxicity

In natural outbreaks, deaths have occurred in livestock, primarily sheep and cattle, when large numbers of sawfly larvae have been ingested. In Australia, outbreaks have occurred in Queensland, where large forests of silver-leaf iron bark trees (*Eucalyptus melanophloia*) provide sustenance for *Lophorotoma interrupta* (Dutra *et al.*, 1997). Large piles of larvae accumulate on the ground under the trees and are ingested by cattle and sheep. In Denmark, a similar outbreak in sheep and goats involving the sawfly *Arge pullata* occurred associated with birch trees (*Betula pendula*) heavily infested with larvae. Outbreaks of sawfly poisoning of cattle, sheep, and pigs in Uruguay were associated with masses of *Perreyia flavipes* crawling through grass in orderly columns. In

Uruguay, sheep were reported to be less frequently affected than cattle. Mortality rates on affected farms have been reported to range from 1.3 to 28% (Dutra *et al.*, 1997).

Minimum lethal dosages of lophyrotomin and pergidin when dosed intraperitoneally in C57/B16 male mice were 8 and 32 mg/kg, respectively (Oelrichs *et al.*, 2001). Administration of intact *P. flavipes* larvae to pigs, calves, and lambs resulted in mortality (Dutra *et al.*, 1997). Deaths occurred in pigs and calves at dosages of 40 g of larvae per kilogram body weight. Sheep administered 10, 20, and 40 g of larvae per kilogram body weight died at 68, 43, and 14 h, respectively. One calf administered 9 g of larvae per kilogram body weight developed diarrhea and became depressed, anorexic, and ataxic but recovered 10 days following dosing. The toxicity of live and dead larvae is equivalent.

Livestock ingesting sawfly larvae may be found dead without prodromal signs (Dutra *et al.*, 1997). Clinically, affected cattle may develop weakness, muscle tremors, depression, agitation, aggression, stupor, and death within 2–7 days. Icterus and mild photosensitization were reported in some cattle that survived. In sheep and goats, clinical signs included depression, anorexia, ataxia, recumbency, and death (Thamsborg *et al.*, 1987).

Clinical laboratory abnormalities associated with sawfly larvae poisoning include elevations in liver enzymes aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and  $\gamma$ -glutamyltransferase (Thamsborg *et al.*, 1996).

Massive hepatic necrosis was the primary postmortem lesion found in cattle and sheep, whereas renal tubular degeneration was occasionally identified in sheep (Thamsborg *et al.*, 1987; Dutra *et al.*, 1997). Lesions secondary to hepatic failure included widespread hemorrhage, icterus, and photosensitization (Dutra *et al.*, 1997).

### Treatment

Management of sawfly larvae poisoning would include removing animals from the source of the larvae and providing symptomatic and supportive care. Administration of silymarin (44–58 mg/kg intravenously) in combination with penicillin (200,000 IU/kg intravenously) and glucose (20 g/lamb) at 7 and 24 h following dosing with sawfly larvae prevented clinical signs of toxicosis and significant liver enzyme changes in experimental lambs (Thamsborg *et al.*, 1996). Lambs receiving larvae along with glucose and penicillin without silymarin developed a milder course of clinical illness and lower liver enzyme changes than did untreated control lambs administered sawfly larvae.



## LEPIDOPTERA: BUTTERFLIES AND MOTHS

Caterpillars of many species of moths and butterflies may possess hairs and spines on their surfaces that can trigger urticarial reactions in humans and animals (Mullen, 2002a). Necrosis and partial sloughing of the tongue was reported in three dogs in Israel following exposure to the pine processionary moth (*Thaumetopoea wilkinsoni*); early signs included vomiting, swelling of the tongue, dyspnea, hyperthermia, tachypnea, cyanosis, lingual edema, labial angioedema, ptyalism, bilateral submandibular lymphadenopathy, and conjunctivitis (Bruchim *et al.*, 2005). All dogs survived following symptomatic care. Caterpillars of monarch butterflies that feed on milkweed plants may accumulate the cardiotoxic glycosides from the plant into their bodies (Fowler, 1993). Upon metamorphosis into monarch butterflies, the glycosides are localized to the wings, and predatory animals such as birds, lizards, and small mammals may be poisoned if they ingest the monarchs.

## PHASMATODEA: WALKING STICKS

Ulcerative keratitis has been reported when dogs encountered the two-striped walking stick (*Anisomorpha buprestoides*). These insects are distributed through Florida and the Gulf coastal plain west to Texas, and they possess defensive glands that produce a lachrymogenic and irritation substance. When annoyed, the walking stick will spray this secretion at its harasser (Dziezyc, 1992).

## AMPHIBIA, ANURA: TOADS

### Introduction

Poisonous amphibians include frogs, salamanders, and toads, but only toads have been associated with toxicosis in domestic animals. Poisonous frogs belong to the Dendrobatidae family and are popular aquarium pets due to their vivid, gem-like coloration. Poisonous frogs have been found to have nearly 500 different bioactive alkaloids in their skin secretions, some of which have been used for food procurement by Central and South American aboriginals who live in areas that these frogs inhabit (Daly, 1980; Daly *et al.*, 2002; Takada *et al.*, 2005). Most of these alkaloids are not thought to be synthesized *de novo* by the frogs but, rather, originate from insects that make up the frogs' diets; the alkaloids are absorbed from the insects and sequestered in the glands

to produce the skin secretions (Daly, 1995a,b; Donnelly, 2002; Saporito *et al.*, 2004). Captive dendrobatids tend to lack these toxic alkaloids in their skin secretions, most likely due to the difference in diets. The remaining discussion focuses on poisonous toads.

### Background

Members of the genus *Bufo* are found throughout the world, and all are considered to produce zootoxins capable of causing clinical effects in animals. However, only the larger toads, specifically *B. marinus*, *B. alvarius*, *B. regularis*, and *B. blombergi*, are considered to produce sufficient poison to cause serious toxicosis (Fowler, 1993). *Bufo marinus* is found in Florida, Texas, Hawaii, the Caribbean, Central and South America, Fiji, Australia, the Philippines, and the Marianas. *Bufo alvarius* is found in the Imperial Valley of California and the Colorado River basin between Arizona and California. *Bufo blombergi* is found only in Colombia, and *B. regularis* is found in Ethiopia (Fowler, 1993).

Toads possess mucous glands, which are widely distributed over the skin and produce a slimy secretion that keeps the skin moist and lubricated. The mucus secretions may aid in defense through their objectionable odor and/or taste to predators. Toads produce their toxic secretions from granular glands, modified mucous glands, throughout the head, shoulders, and dorsolateral areas of their skin. The more toxic toad species possess a parotid gland, which is an aggregation of granular glands located caudal and lateral to the ear (Fowler, 1993).

### Toxicokinetics

Dogs are the species most commonly involved in toad toxicosis. Mouthing of toads stimulates release of toxins from the parotid gland into the mouth, with absorption occurring across the mucous membranes in the mouth (Roder, 2004). Toad secretions contain a variety of compounds, including bufogenins, bufotoxins, and bufotenines.

### Mechanism of action

Bufogenins inhibit sodium–potassium ATPase activity in a manner similar to cardiac glycosides such as digitalis, ultimately causing increased intracellular calcium in myocardial cells that results in cardiac arrhythmias (Eubig, 2001). Bufotoxins are bufogenins conjugated with suberyl arginine, and they have a similar mechanism of action as bufogenins. Bufotenines are indolalkylamines such as serotonin and 5-hydroxytryptophan; in combination with catecholamines present in *Bufo* toxin, these

agents may be responsible for many of the neurologic and gastrointestinal effects of *Bufo* toxins (Eubig, 2001).

## Toxicity

The relative potency of *Bufo* toxins varies with species of toad, geographic location, and size of the toad (Fowler, 1993). Smaller patients may show more severe signs when exposed to toad secretions (Eubig, 2001). *Bufo* toads in Florida appear to be more toxic than those in Hawaii and Texas, based on mortality rates of dogs exposed to the toads (Roder, 2004). One hundred milligrams of crude *Bufo* toxin is said to be toxic for dogs weighing 9–14 kg.

Clinical effects of toad poisoning include hypersalivation, anxiety, and vomiting, which can occur almost immediately following exposure; also, death may occur as rapidly as 15 min following exposure (Eubig, 2001). Other signs, including hyperemic mucous membranes, recumbency, collapse, and tachypnea, may also be present. Neurologic effects are common and include convulsions, ataxia, nystagmus, stupor, or coma (Fowler, 1993; Roder, 2004). A variety of cardiac arrhythmias have been reported, including bradycardia, sinus tachycardia, and sinus arrhythmias (Eubig, 2001). Pulmonary edema, hyperthermia, and hyperkalemia have also been reported (Fowler, 1993).

## Treatment

On-the-spot decontamination of the oral cavity by copious water lavage is recommended in cases in which no signs beyond ptyalism and gagging have occurred (Eubig, 2001). Patients displaying more severe signs should be transported to a veterinary facility for stabilization prior to oral lavage. If an entire toad has been ingested, emesis under veterinary supervision is indicated for dogs when no signs beyond hypersalivation have occurred. Endoscopic or surgical removal of the toad from the stomach may be required in cases in which signs have developed, but it is important to stabilize the patient prior to attempts to remove the toad. Alternatively, multiple doses of activated charcoal with a cathartic may be used when entire toads are ingested (Eubig, 2001).

Patients displaying severe signs of toxicosis should be treated symptomatically and aggressively. Seizures may be managed with diazepam or a barbiturate. Intravenous fluid therapy is essential to aid in cardiovascular support. Arrhythmias should be managed as they develop. Bradycardia may be treated using atropine, whereas propranolol or esmolol may be used to treat tachycardia (Eubig, 2001). Correction of potassium imbalances should be performed as needed. Animals experiencing severe hyperkalemia, severe neurologic signs, or severe

arrhythmias unresponsive to therapy may be treated with digoxin-specific antigen-binding fragments (digoxin immune Fab); however, the high cost of this product often makes its use in veterinary medicine unfeasible (Eubig, 2001).

The prognosis for patients exposed to small toads and/or showing mild clinical signs is good. Animals developing advanced neurologic or cardiac signs have a more guarded prognosis.

## REPTILIA

Venomous reptiles come from the order Squamata, which includes the lizards and the snakes. Outbreaks of poisonings from consumption of the flesh of certain turtles and tortoises have occasionally been reported in humans, but the incidence is fairly low; reports in the veterinary literature of chelonitoxism are lacking.

## SNAKES

### Introduction

Of the 35,001 species of snakes in the world, approximately 400 are venomous (Russell, 2001). Venomous snakes are widely distributed throughout the world, with the exception of certain islands, such as Hawaii, Ireland, and New Zealand (Fowler, 1993). Venomous snakes come from the families Colubridae, Crotalidae, Elapidae, Hydrophiidae, Laticaudidae, and Viperidae; only members of the Crotalidae and Elapidae are represented in the Western Hemisphere.

## CROTALIDS

### Background

The crotalids are also known as pit vipers, so named for the indented, heat-sensing pits located between the nostrils and eyes. Other features of this family include elliptical pupils, triangular-shaped heads, retractable and hollow front fangs, and a single row of subcaudal scales distal to the anal plate (Peterson, 2004). Rattlesnakes have special keratin “rattles” on the ends of their tails and are members of the genera *Crotalus* and *Sistrus*. Other crotalids native to North America include water moccasins, also known as cottonmouths (*Agkistrodon piscivorus*) and copperheads (*A. contortrix*).

At least 29 subspecies of rattlesnakes are found throughout the United States, whereas copperheads and water moccasins are distributed through the eastern and central United States. The approximate distributions of some of the more common species are illustrated in Figure 73.3. Because of their tendency to associate near areas of human habitation, copperheads are responsible for the majority of human (and likely animal) snakebites in the United States. However, the majority of animal and human deaths in the United States are attributed to rattlesnakes, due to their more potent venoms. It is estimated that from 150,000 to 300,000 animals are bitten every year by pit vipers within the United States (Peterson, 2004; Wallis, 2005).

### Toxicokinetics

Pit vipers inject their venom by rotating their retractable fangs downward and forward in a stabbing motion (Peterson, 2004). Contraction of muscles in the venom glands then forces the venom through the hollow fangs and into the tissues of the victim. Snakes can control the amount of venom delivered to the victim by regulating the muscular contraction of the venom glands.

### Mechanism of action

Crotalid venoms are complex combinations of enzymes, cytotoxins, neurotoxins, cardiotoxins, hemolysins, coagulants/anticoagulants, lipids, nucleosides, nucleotides, organic acids, and cations such as zinc (Fowler, 1993; Peterson, 2004). Most venoms contain a minimum of 10 of these different components. In North American rattlesnakes, three general venom types have been defined (Peterson, 2004). "Classic" diamondback venom causes marked tissue destruction, coagulopathy, and hypotension. Mojave A venom causes little tissue destruction or coagulopathy but causes severe neurotoxicosis. The third venom class contains components of both classic and neurotoxic venoms.

Hyaluronidase ("spreading factor") and other enzymes break down collagen and other connective tissues, allowing for rapid penetration of venom components throughout the victim's tissues. Low-molecular-weight myotoxins open sodium channels in the muscle cell membrane, leading to myocyte necrosis. Phospholipase A stimulates hypercontraction of myocyte membranes, resulting in myofibril rupture (Peterson, 2004). Alteration of blood coagulation may lead to either hyper- or hypo-coagulation through either direct effects on clotting factors or induction of hyperfibrinolysis, resulting in dissolution of clots as they are forming. Some diamondback rattlesnake venoms contain cardiotoxic agents

(myocardial depressant factors) that cause profound hypotension unresponsive to fluid therapy. Neurotoxic components bind the presynaptic nerve membrane, inhibiting neurotransmitter release and causing paralysis (Fowler, 1993).

### Toxicity

Dogs are the domestic species most commonly bitten by pit vipers. In a study of prairie rattlesnake bites in dogs, most bites occurred between May and September during the late afternoon (Hackett *et al.*, 2002). Most bites involved young dogs (median age, 3.7 years) and were located on the head.

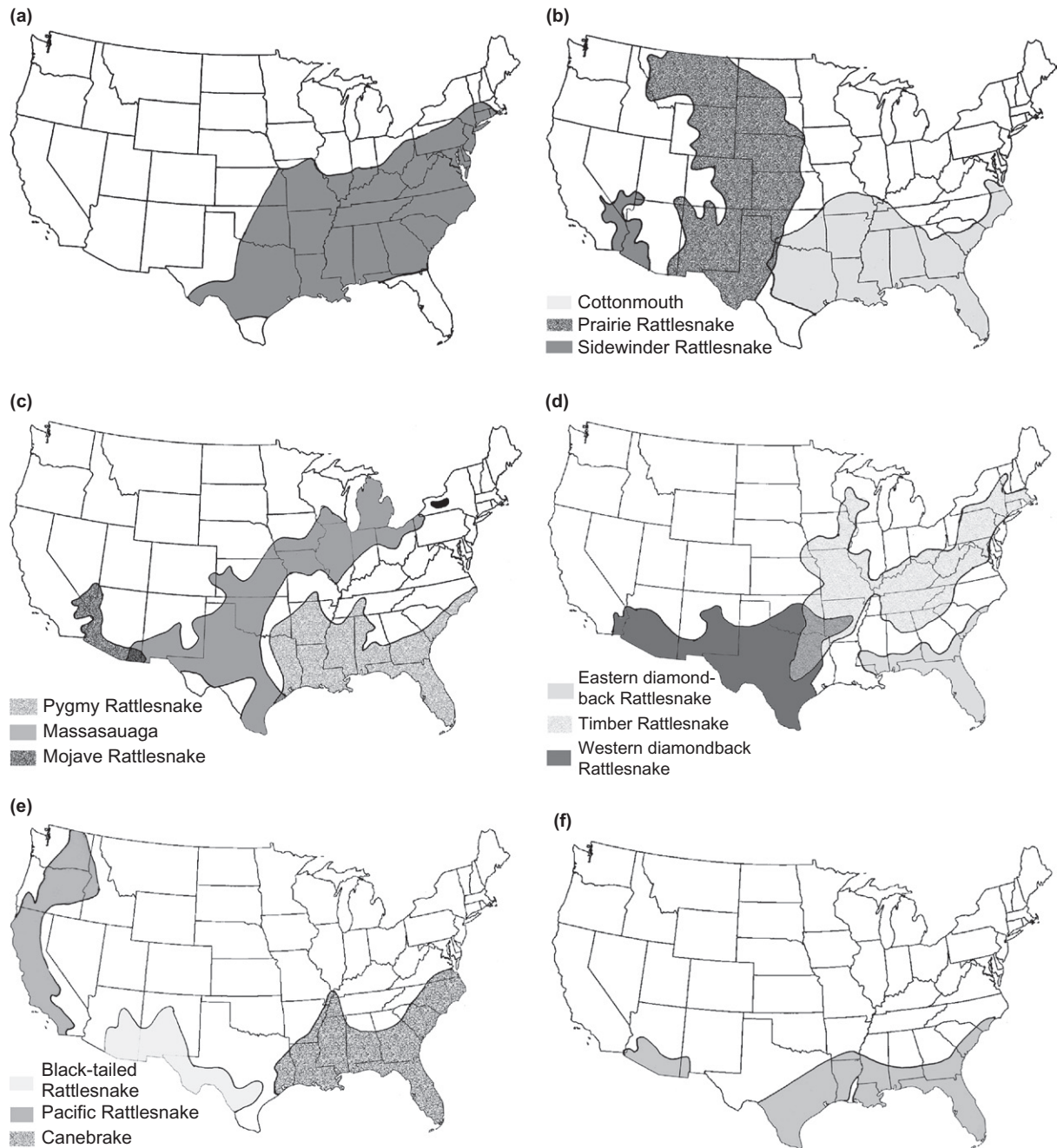
Dogs are most commonly bitten on the front legs and head, horses are most commonly bitten on the muzzle, and cattle are more commonly bitten on the tongue or muzzle (Peterson, 2004). Cats are considered more resistant to pit viper venom, but cats are often presented in more severe clinical conditions due to their small body size, tendency to be struck on the torso, and, often, delay in obtaining veterinary care when they run and hide following a bite. Bites to the thoracic wall allow for more rapid and complete venom absorption, whereas bites to the tongue are essentially equivalent to intravenous envenomations.

The toxicity of any given crotalid bite will depend on both victim and snake factors. The species of the victim is an important factor because some species of animals have a degree of natural immunity to snake venoms. In addition, the victim's size, pre-existing health, interval until medical care is obtained, and site of the bite are victim-related factors that can influence the severity of the snakebite. Snake factors that affect toxicity include species, size and age of the snake, as well as the time of year, time since the snake's last bite, and whether the bite was defensive or offensive. "Dry" bites, those in which no venom is injected, may occur in up to 25% of snakebites (Peterson, 2004). Snakes that have recently fed may be depleted of venom and thus inject less (Fowler, 1993). Older snakes may have more potent venom, and larger snakes may harbor larger volumes of venom to deliver.

Analysis of venoms from a variety of North American pit vipers indicates that the ranking of relative toxicity of venoms, from highest to lowest, is Mojave A rattlesnake (*Crotalus scutulatus*), eastern diamondback (*C. adamanteus*), western diamondback (*C. atrox*), timber rattlesnake (*C. horridus*), cottonmouth (*A. picivorus*), and copperhead (*A. contortrix*).

### Clinical effects

In most cases of snakebite, the initial signs are usually local pain and swelling, followed by petechiation,



**FIGURE 73.3** Distribution of venomous snakes in the United States. (a) Distribution of copperheads (*Agkistrodon contortrix*). (b) Distribution of cottonmouths (*Agkistrodon piscivorus*), prairie rattlesnakes (*Crotalus viridis*), and sidewinder rattlesnakes (*Crotalus cerastes*). (c) Distribution of pygmy rattlesnakes (*Sistrurus miliarius*), massasauga rattlesnake (*Sistrurus catenatus*), and Mojave rattlesnake (*Crotalus scutulatus*). (d) Distribution of the Eastern diamondback rattlesnake (*Crotalus adamanteus*), timber rattlesnake (*Crotalus horridus*), and Western diamondback rattlesnake (*Crotalus atrox*). (e) Distribution of the black-tailed rattlesnake (*Crotalus molossus*), Pacific rattlesnake (*Crotalus atrox*), and canebrake rattlesnake (*Crotalus horridus atricaudatus*). (f) Distribution of coral snakes (*Micruroides euryxanthus* and *Micrurus fulvius*). (Adapted from Fowler, *Veterinary Zootoxicology*, CRC Press, and Professional Pest Control Products website, <http://www.pestproducts.com/snakeinfo.htm>.)

ecchymosis, and discoloration of the skin in the region of the bite. Swelling and/or the haircoat of the victim may mask bite wounds. Bites from snake species that possess only neurotoxic venom may show little local swelling.

Several hours may elapse before more significant clinical signs develop. Hypotension, shock, tachycardia or other arrhythmia, vomiting, diarrhea, shallow respiration, lethargy, nausea, obtundation, muscle fasciculations,



ptyalism, and pain and enlargement of regional lymph nodes may occur (Peterson, 2004). Local tissue necrosis may be severe, especially in areas with minimal muscle mass. Local swelling from bites to the head or front legs in dogs may result in regional circulatory alterations that reduce venom uptake (Peterson, 2004). Horses or llamas bitten on the muzzle may succumb to asphyxiation due to occlusion of the nares due to tissue swelling. Horses develop pitting edema that frequently progresses to involve the entire head and neck or limb, but tissue necrosis is uncommon (Fowler, 1993). Hemolysis, rhabdomyolysis, thrombocytopenia, and coagulopathy may occur.

## Treatment

Historically, a variety of first aid measures have been described for managing snakebites in humans and animals, including suction, electric shock, ice packs, and tourniquets (Bush, 2004; Peterson, 2004). However, most of these measures are of questionable value, and they waste precious time that could be used to transport the patient to a veterinary medical facility. Initial first aid should be focused on keeping the animal quiet (exertion will hasten the spread of venom factors into the tissues) and attempting to keep the bitten area below heart level.

The haircoat around the bite area should be clipped in order to fully visualize the bite. Use of a permanent marker to delineate the margins of local edema and/or circumferential measurements above, at, and below the bite site will allow objective evaluation of the progression of local tissue involvement (Fowler, 1993; Peterson, 2004). Vital signs, including blood pressure, should be monitored closely for the first several hours; all patients should be monitored for a minimum of 24h before being released (Fowler, 1993). Tracheostomy may be required in cases in which severe swelling in the head or neck region results in respiratory compromise or in cases of obligate nasal breathers (e.g., horses and llamas) that are bitten on the muzzle. Intravenous crystalloid therapy is recommended to manage hypotension or hypovolemia. Coagulopathy and hemolysis should be managed using blood or platelet transfusions. Conditions such as muscle tremors, seizures, cardiac arrhythmias, and pulmonary edema should be managed as they develop using standard medical therapies. Corticosteroid use is controversial in snakebites, with some indicating that corticosteroids have no place in management of snakebite (Peterson, 2004), whereas others suggest that judicious use of corticosteroids may be of benefit (Fowler, 1993). In horses, topical application of dimethyl sulfoxide to the bite site should be avoided because it enhances systemic absorption of venom (Peterson, 2004). Diphenhydramine may be useful for its sedative effect. Broad-spectrum antibiotics are advocated by some to aid in prevention of

infection (Peterson, 2004), and horses should receive tetanus antitoxin or toxoid (Fowler, 1993).

The use of intravenous antivenin in crotalid snakebites can result in the reversal of potentially life-threatening problems such as coagulopathy, thrombocytopenia, and paralysis (Peterson, 2004). Antivenin cannot reverse tissue necrosis or secondary effects such as renal damage. One study on prairie rattlesnake bites in dogs suggested that antivenin administration was not significantly associated with clinical outcome, and the value of its use in prairie rattlesnake envenomation was questioned (Hackett *et al.*, 2002).

Equine-origin polyvalent antivenin has historically been used to manage snakebites in animals. More recently, polyvalent immune Fab fragments (CroFab and Protherics) have become available to treat snakebites in humans and animals. Advantages of the crotalid Fab antivenin over the equine-origin antivenin include decreased antigenicity (and therefore decreased potential for allergic reaction), more rapid elimination, increased stability, and more rapid reconstitution (Gwaltney-Brant and Rumble, 2002). The crotalid Fab antivenin has been shown to be effective in management of canine snakebite (Peterson, 2004). Administration of either antivenin should be begun as early as possible, and patients should be closely monitored for signs of anaphylaxis during antivenin administration. Studies in dogs have shown that single vial doses of crotalid Fab antivenin are sufficient to manage most cases of canine snakebite envenomation. Coagulation parameters should be monitored, and additional vials of antivenin should be administered if deterioration is noted.

The prognosis for recovery from snake envenomation is dependent on the type of snake involved, the severity of the envenomation, and the rapidity and aggressiveness of veterinary intervention.

A rattlesnake vaccine (*Crotalus Atrox* Toxoid, Red Rock Biologics) has been developed and marketed with the aim of reducing the morbidity and mortality of dogs due to crotalid snakebites (Wallis, 2005). The vaccine is designed to elicit an immune response to the major protein fractions of *Crotalus atrox* (western diamondback rattlesnake) that may also cross-react to the major protein fractions of some other rattlesnake venoms. The vaccine does not induce neutralizing antibodies against Mojave neurotoxin. The vaccine is administered as two injections (three for dogs exceeding 100 pounds of body weight) spaced 4 weeks apart. Titers peak in 4–6 weeks and decline after approximately 6 months, and twice-yearly vaccination is recommended for dogs that have potential to be exposed to rattlesnakes year-round. The manufacturer emphasizes that the vaccine does not eliminate the need to seek veterinary care in the event that a venomous snake bites a vaccinated dog. In clinical trials, vaccinated dogs experiencing mild envenomations from

subsequent snakebites frequently had their swelling receding within hours of the bite, compared to unvaccinated dogs, which may have swelling progress and persist for 1 or 2 days following a mild envenomation. Vaccinated dogs that are severely envenomated may still require treatment with antivenom, although the amount of antivenom required may be reduced in vaccinated dogs (Wallis, 2005). The vaccine has been on the market for only a few years, and additional time and study are required to determine its overall efficacy and value.

## ELAPIDS

### Background

In North America, there are two species of venomous elapid snakes: the Sonoran coral snake (*Micruroides euryoxanthus*) and several subspecies of *Micrurus fulvius*, including the Texas coral snake (*M. f. tenere*), the eastern coral snake (*M. f. fulvius*), and the south Florida coral snake (*M. f. barbouri*) (Peterson, 2004). The Sonoran coral snake is found in central and southeastern Arizona and southwestern New Mexico. The Texas coral snake is found from eastern and south-central Texas north into Louisiana and southern Arkansas. The eastern coral snake is present from eastern North Carolina south to central Florida and west through Alabama and Mississippi. The south Florida coral snake is located in southern Florida.

North American coral snakes tend to be shy, nonaggressive, and nocturnal, making interactions between them and domestic animals less common than interactions with pit vipers (Peterson, 2004). These snakes are brightly colored, with alternating bands of black, red, and yellow, and they have small heads and round pupils. The venom delivery apparatus of coral snakes includes short, fixed (nonhinged) front fangs that are partially covered by a membrane (Fowler, 1993). During the bite, the membrane is pushed away and the venom duct empties at the base of the fang, bathing the fang with venom that runs down grooves in the fang. During the bite, coral snakes hold onto the victim and chew, delivering additional venom to the wound.

### Toxicokinetics

Like crotalid venom, coral snake venom is composed of a variety of compounds, mostly small polypeptides and enzymes. Neurologic signs following envenomation may be delayed in onset for up to 12 h, and the duration of effects is prolonged (Peterson, 2004). Total clearance of venom from the body may take up to 14 days.

### Mechanism of action

Neurotoxic peptides in coral snake venom cause a nondepolarizing postsynaptic neuromuscular blockade similar to the effects of curare (Peterson, 2004). Binding of neurotoxins to postsynaptic receptors appears to be irreversible. Enzymes within the venom can cause local tissue necrosis, myoglobinemia in cats, and hemolysis in dogs.

### Toxicity

As with crotalid envenomations, the severity of the bite is related to the size of the victim and the amount of venom delivered. The amount of venom injected is related to the duration of the bite, intensity of chewing, and reason for the bite (offensive versus defensive). Clinical signs vary with the species of the victim. In cats, the signs are primarily neurologic, including progressive ascending flaccid paralysis, decreased nociception, CNS depression, and diminished spinal reflexes (Peterson, 2004). Hypotension, respiratory depression, anisocoria, myoglobinemia, and hypothermia have also been described in cats. In dogs, depression of the CNS, decreased spinal reflexes, muscle weakness, and respiratory depression may occur. Vomiting, hypersalivation, hypotension, dyspnea, dysphagia, muscle fasciculation, tachycardia, and hemolysis have also been reported in dogs. Potential complications include dysphagia leading to aspiration pneumonia. Death is due to respiratory paralysis.

### Treatment

Management of coral snake envenomations in animals should entail immediate transport for veterinary care. Because onset of clinical signs may be delayed up to 12 h, patients that have been bitten should be closely monitored for a minimum of 24 h. Baseline serum chemistry and complete blood count values should be obtained, and respiratory function should be closely monitored. Ventilatory support may be required if clinical signs progress to the point where respiration is compromised; ventilation may be required for 72 h or more (Peterson, 2004). Administration of specific *Micrurus* antivenin should be considered if neurologic signs begin to develop; early administration is recommended because the antivenin is considered poorly effective at displacing venom components bound to receptor sites (Peterson, 2004). As with crotalid antivenin, anaphylaxis is a potential complication of *Micrurus* antivenin administration. Broad-spectrum antibiotics and symptomatic wound care may be indicated. Recovery periods of 7–10 days

have been reported in cats envenomated by coral snakes (Chrisman *et al.*, 1996).

The prognosis for coral snake envenomations in animals is generally good provided prompt and aggressive veterinary care is obtained.

LIZARDS

Background

Venomous lizards are found only in North and Central America and are members of the genus *Heloderma*. *Heloderma suspectum* and *H. cinctum* are commonly referred to as Gila monsters, whereas *H. horridum* is known as the Mexican beaded lizard. The Gila monsters are found in Arizona and areas of Utah, New Mexico, Nevada, and Southern California, whereas the Mexican beaded lizard is found in Mexico from Sonora to the Pacific Coast and into Guatemala (Cantrell, 2003; Peterson, 2004).

Venomous lizards are large and heavily bodied, with blunt, rounded tails, powerful jaws, and short legs with clawed, hand-like feet. The colorful, bead-like scales form a reticular pattern of dark brown to black on a yellow, orange, pink, and/or cream background. Gila monsters, which can reach 55 cm in length, are smaller than Mexican beaded lizards, which can grow up to 1 m in length and weigh up to 2 kg (Cantrell, 2003; Peterson, 2004).

*Heloderma* spp. possess venom glands in the lower jaw at the base of the teeth, and venom is delivered through grooves in the teeth via capillary action as the lizard masticates. These lizards are generally considered to be docile, but when provoked they can be aggressive biters that can hang on tenaciously to their victim, often requiring prying the jaws open to release the victim (Cantrell, 2003). The teeth are loosely attached and brittle, and they may break off into the wound. The venom is considered a defensive weapon rather than one for procuring food (Peterson, 2004).

Mechanism of action

*Heloderma* venom is composed of a complex mixture of proteins and enzymes, many of which are similar to those found in snake venoms, including hyaluronidase, phospholipase A<sub>2</sub>, serotonin, and a variety of enzymes (Cantrell, 2003). Hyaluronidase (“spreading factor”) catalyzes the cleavage of internal glycoside bonds of acid mucoglycosides, resulting in decreased viscosity of hyaluronic acid, which in turn increases tissue permeability and allows deeper penetration of venom into tissue. Phospholipase A<sub>2</sub> uncouples oxidative phosphorylation, which inhibits cellular respiration, causes cell

TABLE 73.3 Contents and effects of various *Heloderma* spp. venom components

Component	Effects	References
Gilatoxin	“Lethal factor,” kallikrein-like activity, pain, hypotension	Fowler (1993), Utainsincharoen <i>et al.</i> (1993)
Helodermin	Vasodilation, hypotension	Grundemar and Hogestatt (1990)
Helospectin I and II	Vasodilation, hypotension	Grundemar and Hogestatt (1990)
Helothermine	Lethargy, paresis, hypothermia	Peterson (2004)
Hyaluronidase	“Spreading factor,” degrades connective tissue	Peterson (2004)
Phospholipase A <sub>2</sub>	Inhibition of platelet aggregations	Huang and Chiang (1994)

membrane destruction, and inhibits platelet aggregation (Huang and Chiang, 1994; Peterson, 2004). Various proteolytic enzymes result in local tissue damage as well as aid in the spread of venom through the tissue. In addition, *Heloderma* venoms contain several unique components that have a variety of clinical effects; these are summarized in Table 73.3. Gilatoxin is considered to be the major lethal factor in *Heloderma* venom (Fowler, 1993).

Toxicity

The toxicity of *Heloderma* venoms is dependent on the amount of venom delivered to tissues, which in turn is dependent on the duration and severity of the bite. Due to their inquisitive nature and tendency to harass wildlife that they encounter, dogs are the species most likely to have a significant encounter with *Heloderma* spp. Although rare, *Heloderma* bites to dogs and cats have occurred, and the death of at least one dog has been reported (Fowler, 1993; Peterson, 2004).

The clinical effects of *Heloderma* envenomation include intense local pain, edema, and hemorrhage at the site of the wound (which may contain fractured teeth). Regional lymphangitis and local ecchymoses may occur, although tissue necrosis is not common. Systemic signs described in humans include weakness, dizziness, tinnitus, muscle fasciculations, hypotension, and tachycardia. In dogs and cats, signs may include tachypnea, vomiting, polyuria, salivation, and lacrimation. Aphonia has been reported in cats (Peterson, 2004). Hypotension, tachycardia, and respiratory distress have been reported in dogs and cats injected with boluses of *Heloderma* venom, although reports of these conditions in natural exposures are lacking.

## Treatment

Management of *Heloderma* bites in small animals includes initial first aid followed by assessment and monitoring. The first course of action is often disengaging a lizard that is still attached to the victim because these lizards may hold on tenaciously once they bite. Removal should be attempted as quickly as possible because the amount of venom delivered is proportional to the duration of the bite. Suggested means of removal of the lizard include applying a flame from a match or cigarette lighter to the underjaw of the lizard, prying the jaws open with a metal bar, or killing the lizard by incising the jugular vein with a knife. Attempts to pull the lizard off the victim or to strike the lizard in the head may enlarge or deepen the wounds at the site of the bite (Fowler, 1993). Once the lizard is removed, a veterinarian should evaluate the patient. The bite site should be irrigated with 2% lidocaine and the wound probed with a 25-gauge needle to detect any embedded tooth fragments (Peterson, 2006). Diazepam, 0.1–0.5 mg/kg intramuscularly, may be helpful in sedating agitated or highly painful animals. Analgesics should be administered as needed, and the animal should be monitored for several hours for the development of hypotension or shock; if either develops, appropriate medical treatment including intravenous fluid therapy is indicated. Broad-spectrum antibiotics should be administered to prevent infection from the myriad of potentially pathogenic bacteria that have been reported to frequent the mouth of reptiles (Peterson, 2006). General wound care should be instituted, and the wound should be examined daily for evidence of infection. Envenomation of pets by *Heloderma* spp. is rare, and in most cases, a favorable outcome can be expected provided prompt and appropriate veterinary care is obtained.

## AVES

Two genera of passerine birds native to New Guinea, *Pitohui* and *Ifrita*, have poisonous secretions on their feathers, most likely as means of defense against predators (Dumbacher *et al.*, 1992; Weldon, 2000; Dumbacher and Fleischer, 2001). The toxins are present in highest concentrations in the contour feathers of the belly, breast, and legs, with lesser amounts on the head, back, tail, and wing feathers (Weldon, 2000). Several steroidal alkaloids have been isolated from the feathers of these birds, including batrachotoxin and homobatrachotoxin as well as derivatives of these compounds; similar compounds are present on the skin of poison dart frogs (Dendrobatidae) from Central and South America (Daly, 1995b).

The origin of the toxins found in *Pitohui* ("rubbish bird") and *Ifrita* ("bitter bird") is thought to be through accumulation from insects ingested by the birds rather than by *de novo* synthesis (Dumbacher *et al.*, 2004). High levels of batrachotoxins have been found in beetles of the genus *Choresine* (family Melyridae) that serve as part of the diet of *Pitohui* species. The beetles themselves are unable to synthesize steroidal skeletons and likely accumulate the base steroids from plant phytosterols that they ingest. The beetles then convert the steroids to the steroidal alkaloids and accumulate them within their bodies.

Batrachotoxins act through the opening of sodium channels in nerve and muscle cells, resulting in depolarization and paralysis (Dumbacher *et al.*, 2004). However, exposure of humans to the feathers of *Pitohui* and *Ifrita* species primarily results in respiratory irritation; allergic-type responses; and burning sensations of the eyes, skin, and oral mucosa (Dumbacher *et al.*, 2000).

## MAMMALIA

Only a handful of mammals are poisonous or venomous, and toxicosis from exposure to these animals is uncommon. Poisonous mammals include certain carnivores (e.g., polar bears) and pinnipeds (e.g., walrus and seals), whose livers contain high levels of vitamin A; ingestion of these livers may result in acute or chronic hypervitaminosis A (Cleland and Southcott, 1969; Fishman, 2002). In addition, the meat of marine mammals such as whales may accumulate high levels of organotins, mercury, and other agents that can pose a toxicologic hazard if ingested in sufficient quantity (Endo *et al.*, 2005). Impairment in cell-mediated immunity was found in sled dogs from Greenland that were fed the blubber of minke whales (*Balaenoptera acutorostrata*) (Sonne *et al.*, 2006).

Venomous mammals belong to the orders Monotremata and Insectivora (Fowler, 1993). Venomous monotremes are the platypus and echidna, of which only the platypus is considered to be of toxicologic significance because the venom gland in the echidna is nonfunctional. The male platypus possesses a curved, grooved, conical, sharp spur on the medial aspect of the tarsus. The spur is normally kept retracted against the leg, but when stimulated, muscles cause the spur to project perpendicularly to the leg. The venom gland is kidney shaped and located on the medial aspect of the thigh, and venom is first transported to a reservoir near the spur and then moved through a duct to the spur. Envenomation occurs when the platypus kicks the spur into the victim. Although poorly studied, components



identified in platypus venom include a protein similar to natriuretic peptide, a defensin-like peptide, a hyaluronidase, 5-hydroxytryptophan, and histamine (Hodgson, 1997; Torres *et al.*, 2006). Based on reports of human envenomation by platypus, expected signs include intense pain and numbness at the site of the wound and local swelling that may progress proximally (Fowler, 1993). Regional lymph nodes may become enlarged and painful. Humans have reported a feeling of faintness. No reports of human or animal fatality from platypus exist. There are infrequent anecdotal reports of dogs being envenomated by platypus.

Venomous insectivores include various shrews and solenodons and possibly some moles and hedgehogs (Dufton, 1992). The European hedgehog (*Erinaceus europaeus*) has been noted to mix its frothy saliva with toad toxins and smear this mixture over its forward spines. Some moles will store "paralyzed" worms and slugs away, with the paralysis thought to be due to paralytic toxins within the mole's saliva. Studies of the American short-tailed shrew (*Blarina brevicauda*), the Haitian solenodon (*Solenodon paradoxus*), the European water shrew (*Neomys fodiens*), and the Mediterranean shrew (*N. anomalus*) have obtained more definitive evidence of the presence of venoms. These mammals possess three pairs of salivary glands: parotid, retrolingual, and submaxillary. The submaxillary glands are thought to contribute to the bulk of the venom in these species. The Haitian solenodon has grooved incisors that direct the venom to the wound, whereas the shrews have teeth with concave inner surfaces that may play a similar role as grooves.

Envenomation is achieved by biting and chewing the victim, accompanied by copious salivation. Insectivore venoms contain a variety of peptides and enzymes, and a compound isolated from *Blarina* venom has been shown to have kallikrein-like activity (Kita *et al.*, 2005). The venom of *Blarina* appears to be the most toxic, with that of *N. fodiens*, *N. anomalus*, and *Solenodon* being 1/3, 1/6, and 1/20 as toxic, respectively (Dufton, 1992). There is considerable species variation in sensitivity to the venom in shrew and solenodon venom, with voles and rabbits being most sensitive to the effects and mice, cats, and humans being relatively more resistant (Dufton, 1992). In most natural exposures, the primary effects of shrew and solenodon venoms are related to neurotoxicity. Mice bitten by *Blarina* rapidly develop depression and immobility. When submaxillary gland extracts are injected into experimental animals, depression, irregular respiration, dyspnea, ptosis, rear limb paralysis, convulsions, and death occur. Intravenous injection of 7 mg/kg of *Blarina* toxin extract in a cat resulted in decreases in respiratory and heart rates with a concomitant decrease in blood pressure. These effects resolved within 15 min but returned with subsequent doses of 2.5 and 7 mg/kg. Apparent recovery following the final dose was followed

by acute respiratory depression and cardiac arrhythmia, and the cat subsequently died. In humans, symptoms of bites from shrews or solenodons result in localized burning sensation surrounding the lower jaw tooth puncture sites and localized swelling. The burning sensation may persist for several days. Systemic toxicosis has not been reported in natural exposures to the bites of shrews or solenodons in humans or domestic animals.

## CONCLUSIONS

Every phylum of the Animal Kingdom contains animals capable of producing toxic effects, either through envenomation or through poisoning. However, only a small number of these animals are sufficiently toxic to be of importance to veterinary clinicians. Arachnids, insects, toads, and snakes cause the most clinically significant problems for domestic and wild animals. Although in some cases specific antidotes (i.e., antivenins) exist for exposures to venomous animals, availability and cost of these antidotes often make their use in veterinary medicine unfeasible. Therefore, most cases of zootoxicosis in animals will be managed with symptomatic and supportive care.

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## Mare reproductive loss syndrome

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### INTRODUCTION

In April 2001, an outbreak of abortions and early fetal losses among horse farms in central Kentucky resulted in the loss of more than 4500 equine pregnancies (Sebastian *et al.*, 2008). The losses hit particularly hard within the Thoroughbred industry, in which more than 3500 early and late-term abortions accounted for an overall loss of 17% of all pregnancies, with some farms experiencing more than 60% early fetal losses compared to typical losses of 3–5% during the same time interval in other years (Potter *et al.*, 2005). Losses also occurred in other breeds (including approximately 1000 Paint horse and Quarter horse foals) in Kentucky, and smaller numbers of similar fetal losses were reported in surrounding states of Ohio, West Virginia, Tennessee, Illinois, and Indiana (Cohen *et al.*, 2003b; Bernard *et al.*, 2004; Sebastian *et al.*, 2008). In 2002, a similar outbreak occurred that was approximately one-third that of 2001 (McDowell *et al.*, 2010). The total economic loss to the thoroughbred industry in 2001 was estimated to be more than \$330 million, and the combined 2001–2002 losses to mare reproductive loss syndrome (MRLS), as the disorder became known, were estimated to be approximately \$500 million (Sebastian *et al.*, 2008).

Attempts to identify the etiology of MRLS managed to rule out a number of infectious, nutritional, toxic, and metabolic causes. Mares bred in mid-February were found to be at greatest risk of early term abortions, whereas those bred after April 1 were at much lower risk (Cohen *et al.*, 2005). An epidemiological survey performed in the summer of 2001 revealed a temporal correlation between the presence of eastern tent caterpillars (ETC; *Malacosoma americanum*), the presence

of wild black cherry trees (*Prunus serotina*), the presence of waterfowl, and the practice of feeding hay off the ground among the farms where MRLS was reported (McDowell *et al.*, 2010). During the same time frame in which MRLS-related abortions were being investigated, there were increased reports of fibrinous pericarditis and unilateral uveitis in horses on farms in central Kentucky. Mares experiencing early or late-term abortions from MRLS did not experience any long-term effect on fertility and were able to successfully reproduce in subsequent years (Cohen *et al.*, 2005; Sebastian *et al.*, 2007).

### CLINICAL MANIFESTATIONS

The majority of fetal losses attributed to MRLS were early fetal losses occurring between 48 and 171 days of gestation that were detected during routine reproductive ultrasonographic evaluations (Sebastian *et al.*, 2007). Most mares showed no clinical signs, with less than 5% exhibiting mild colic, abdominal straining, or low-grade fevers 1–3 days prior to fetal loss (Bernard *et al.*, 2004). Examination of affected mares revealed that fetuses were absent, present but dead, present and alive with bradycardia and slow movements (these fetuses later died), or present and alive but surrounded by flocculent and cloudy amniotic fluid (some of these fetuses did survive) (Dwyer *et al.*, 2003). Culture of fetal and placental tissues most consistently resulted in growth of non- $\beta$ -hemolytic streptococci or actinobacilli.

Late-term abortions attributed to MRLS were characterized by abortion at more than 269 days of gestation,



live fetuses with slow heart rates and movements, and fetuses with amniotic fluid changes as described for early term abortions. Some foals were born weak and with signs consistent with fetal hypoxia; the majority of these foals did not survive beyond 4 days (Sebastian *et al.*, 2007). Pathologic lesions found in both early and late-term abortions included placental edema, premature placental separation, placentitis, funisitis, thyroidal follicular dysplasia, and pulmonary lesions consisting of inflammatory infiltration of pulmonary parenchyma and/or alveoli and the presence of squamous epithelial cells within alveoli (Cohen *et al.*, 2003a). Up to 40% of late-term aborted fetuses had no pathologic lesions at necropsy. As with early term abortions, non- $\beta$ -hemolytic streptococci and actinobacilli were most commonly isolated from fetal and/or placental tissues.

An outbreak of fibrinous pericarditis occurred during the same time frame as the 2001 MRLS abortions, with 34 terminal cases reported during May and September of 2001 compared to a normal prevalence of 4 cases per year (Bolin *et al.*, 2005). Horses of all ages, breeds, and sexes were represented in the population of affected horses. Nearly half of the affected horses were 2 years of age or younger, suggesting that younger horses may be at greater risk to develop pericarditis. Affected horses presented with tachycardia, pleural effusion, pericardial effusion, ascites, fever, abdominal pain, respiratory distress, weight loss, and tachypnea. Sudden death was reported in a few cases. Ultrasonographic evaluation revealed effusive and fibrinous pericarditis. Pathologic lesions detected at necropsy included cardiomegaly, fibrinous pericardial effusion, thickening of pericardium with fibrin, and restrictive pericarditis with fibrous tissue deposition on pericardial and epicardial surfaces. Culture of pericardial fluid revealed bacterial involvement in 62% of clinical and terminal cases (Bolin *et al.*, 2005). *Actinobacillus* species were the most commonly isolated bacteria from affected horses, although *Enterococcus faecalis*, *Streptococcus zooepidemicus*, and *Escherichia coli* were occasionally isolated. Most of these bacteria showed relatively broad sensitivity to antibiotics.

During the 2001 MRLS phenomenon in central Kentucky, unilateral uveitis was reported in 40 horses of varying age, breed, and sex (Sebastian *et al.*, 2007). Affected horses developed acute exudative ophthalmitis with corneal edema, exudates within the anterior and posterior chambers, and iris hemorrhages. Affected eyes progressed to blindness and global atrophy. No pathologic organisms were detected on culture, and no histopathological examination of affected globes was performed. Also during this time frame, three cases of *Actinobacillus* meningoencephalitis were diagnosed in young (<4 years of age) horses at necropsy.

In central Kentucky, the majority of cases of MRLS and associated disorders occurred during 2001, with fewer cases reported the following year and even fewer in

2003. Retrospectively, similar abortion storms occurred in Kentucky in the spring of both 1981 and 1982, but no epidemiological studies were conducted. In 2006, abortions in mares in Florida were associated with the presence of large numbers of ETC (Sebastian *et al.*, 2007). Similar episodes of equine abortions were also reported in Australia, associated with processionary caterpillars (*Ochragaster lunifer*) (McDowell *et al.*, 2010).

## MECHANISM OF ACTION

Upon initial study of the MRLS phenomenon, several hypotheses regarding the potential cause of the abortions were presented and investigated. An infectious etiology was considered, but serological and virological studies were consistently negative, and the pathological lesions were not characteristic of ascending infections. The presence of funisitis might suggest a hematogenous route of infection, but funisitis was identified in less than half of the cases of MRLS, and many cases had no evidence of infection by any route (Cohen *et al.*, 2003b,c). The bacterial organisms (*Actinobacillus* spp. and non- $\beta$ -hemolytic *Streptococcus*) that were most commonly isolated from affected tissues are common commensal organisms in the equine oral cavity and alimentary tract and are considered opportunistic, not primary, pathogens (Donahue *et al.*, 2006). Moreover, even these commensal organisms were not consistently isolated from all affected tissues, making their role as potential primary pathogens unlikely.

The lack of identification of infectious causes for MRLS stimulated a search for a potential environmental toxin that might explain the abortions (Taylor, 2002; Sebastian *et al.*, 2007). Toxicants that had been associated with abortions in the past, such as nitrates/nitrites and ergot/fescue, were quickly ruled out as potential etiologies, as were phytoestrogens and mycotoxins (Sebastian *et al.*, 2007). The association between ETC and the development of MRLS led some to hypothesize that the cyanogens from black cherry trees (the ETC's preferred food) may be responsible, and very low levels of cyanide were detected in the heart tissue from some aborted fetuses. However, further tests, including studies exposing pregnant mares to cyanide, failed to support the role of cyanide in MRLS. Nor did exposing mares to mandelonitrile, the primary breakdown product of the major cyanogen of cherry trees, induce significant illness or abortion. Other investigations attempting to link MRLS to exposure to other toxicants, including ammonia, were also unsuccessful.

Pregnant mares exposed to live ETC on pasture at a concentration of 11.3–27.5 larvae/25cm<sup>2</sup> for 6 h per day aborted within several days of exposure (Webb *et al.*, 2004). Another experimental study gavaged

pregnant mares (<102 days of gestation) with homogenized ETC, resulting in abortion in four of five mares, whereas mares administered saline or ETC frass (feces) had no fetal losses. Once the relationship between ETC and MRLS was established, studies were undertaken to determine the mechanism by which ETC induces abortion. Fractionating ETC larvae and administering the various fractions to pregnant mares revealed that the abortifacient fraction is associated with the larval exoskeleton (McDowell *et al.*, 2010). Attempts to induce abortion by feeding gypsy moth (*Lymantria dispar*) larvae, which have similar exoskeletons with numerous setae (hairs), and closely related forest tent caterpillars (*Malacosoma disstria*) demonstrated that neither of these species were abortifacient in mares. This suggested that the presence of setae alone was not sufficient to induce abortion and that some unique component of the ETC exoskeleton appears to play a role in triggering abortions (Sebastian *et al.*, 2007). Sterilization of ETC via irradiation reduced but did not completely eliminate the ETC's abortifacient properties. ETC-induced abortion has been reproduced in pigs (a species with a similar epitheliochorial type of placentation), but goats, rats, and mice appear to be resistant to the abortifacient effects of ETC.

Based on natural and experimental disease, the current hypotheses regarding the potential pathophysiology of MRLS-induced disease are focused on the setae (hairs) of the ETC exoskeleton (Tobin *et al.*, 2004; McDowell *et al.*, 2010). The first hypothesis is that the setae cause disruption of the gastrointestinal mucosal barrier, resulting in subclinical bacteremia with hematogenous spread to the pericardium, uvea, meninges, placenta, or fetus (Tobin *et al.*, 2004; McDowell *et al.*, 2010). The presence of microgranulomas surrounding setal fragments within the gastrointestinal tract of mares and pigs fed intact or fragmented ETC supports this hypothesis. Another hypothesis is that the embolization of setal or other exoskeletal fragments may directly carry opportunistic bacteria to the target tissues. However, the inability to culture bacteria from a large percentage of affected tissues tends to argue against these hypotheses. A final hypothesis is that MRLS is caused by an unidentified toxin that is produced by or carried on the ETC and is toxic to the placenta and/or fetus (Sebastian *et al.*, 2007). A dose-response relationship between exposure to ETC and time of abortion has been described that would support a toxic mechanism (Sebastian *et al.*, 2003).

## TREATMENT AND PREVENTION

Because many of the early term abortions happened abruptly, without clinical signs in the mares, treatment was often not able to be instituted. Mares that had

aborted were often treated with broad-spectrum antibiotics, and subsequent uterine cytological evaluations were normal. No long-term effect on fertility was reported in mares that aborted due to MRLS. Live foals born to mares affected by MRLS were treated with supportive care, oxygen therapy, and antibiotics, but frequently these foals failed to survive (Sebastian *et al.*, 2007). Some horses that developed fibrinous pericarditis recovered following treatment with antibiotics selected based on culture and sensitivity. Standard treatments for unilateral uveitis included systemic and topical antibiotics, anti-inflammatory drugs, atropine, tissue plasminogen activator, mycotoxin binders, and cyclosporine. Response to treatment of unilateral uveitis was poor, and many affected horses lost functional use of the affected eye.

Preventative measures suggested to minimize the effect of ETC on subsequent equine breeding seasons included removal of cherry trees from pastures, spraying of pastures and ETC nests with pyrethrin-based pesticides, and muzzling of mares when on pasture (Potter *et al.*, 2005; Sebastian *et al.*, 2007). These measures resulted in a decline in the incidence of MRLS in subsequent breeding seasons.

## CONCLUSIONS

A syndrome of early and late-term abortions, fibrinous pericarditis, unilateral uveitis, and meningoencephalitis in horses exposed to eastern tent caterpillars was reported in 2001 and 2002 in central Kentucky. The causative factor has been tentatively isolated to the exoskeleton of the caterpillar, with the setae being the exoskeleton component of greatest suspicion. Current hypotheses on pathophysiology include an unidentified abortifacient toxin and subclinical bacteremia with subsequent seeding of tissues with opportunistic bacteria.

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# Chemical-induced estrogenicity

Stephen H. Safe, Shaheen Khan, Fei Wu, Xiangrong Li and Sandeep Sreevalsan

## INTRODUCTION

17 $\beta$ -Estradiol (E2) and related steroidal hormones play an important role in multiple physiological processes. However, these hormones are risk factors for hormone-dependent diseases including breast and endometrial cancer. Inappropriate exposures (high or low) to estrogens can also lead to adverse health effects. The identification of estrogenic compounds in the environment, coupled with human exposures to these compounds, has generated public, regulatory, and scientific concern regarding their potential hormonal toxicity to humans and wildlife. This chapter focuses on chemical-induced estrogenicity and the potential toxicological impacts of these compounds.

## ESTROGENS PLAY A ROLE IN NORMAL PHYSIOLOGICAL FUNCTION AND DISEASE

### Background

Estrogens regulate the development and function of the female reproductive system, and their effects are mediated through estrogen receptor  $\alpha$  (ER $\alpha$ ) and ER $\beta$ . However, estrogens are by no means only female hormones. In both males and females, estrogens play crucial physiological roles, including sex differentiation, bone development and maintenance, and central nervous system and cardiovascular function. The ER is important for mammary gland development, and ER $\alpha$

knockout ( $\alpha$ ERKO) mice are viable but sterile and are estrogen-insensitive in several estrogen target organs, including mammary glands, reproductive tracts, and gonads. The well-recognized estrogenic responses, such as uterine weight increase and vaginal cornification, are all absent in  $\alpha$ ERKO mice (Lubahn *et al.*, 1993; Korach, 1994). In  $\alpha$ ERKO mice, mammary glands undergo normal development in the prenatal period, but they fail to develop terminal end buds and are severely undertropic during adulthood (Lubahn *et al.*, 1993; Korach, 1994). Surprisingly, adult male  $\alpha$ ERKO mice are also infertile because of impaired spermatogenesis (Eddy *et al.*, 1996). In contrast to the extensive reproductive tract abnormalities in  $\alpha$ ERKO mice, ER $\beta$  knockout mice are fertile but exhibit reduced fertility (Korach, 1994; Krege *et al.*, 1998).

### Estrogens and women's health

Women have significantly lower risk of cardiovascular disease than do men, and this has been putatively attributed to the beneficial effects of estrogen on cardiovascular function (Nathan and Chaudhuri, 1997). Estrogen decreases low-density lipoprotein levels and increases high-density lipoprotein levels. Estrogen exhibits vasodilatory effects and protects against vascular injury (Pare *et al.*, 2002), and it also induces COX-2-dependent up-regulation of atheroprotective prostacyclin PGI<sub>2</sub> (Egan *et al.*, 2004). In epidemiological studies, estrogen has been linked with improved cardiovascular functions (Grodstein *et al.*, 1996). Bilateral ovariectomy before menopause elevates the risk for coronary heart disease, and the increased risk is eliminated by estrogen replacement therapy (Colditz *et al.*, 1987). However, a large



clinical trial conducted by the Women's Health Initiative reported contradictory results on the effects of estrogens because women taking hormone replacement therapies (HRTs) have an increased risk for heart diseases and stroke (Rossouw *et al.*, 2002). The relationships between estrogen and cardiovascular diseases require further assessment.

Estrogen is also important for bone development and maintenance (Cutler, 1997) and for sexual differentiation in the brain and reproductive behavior (Bakker *et al.*, 2003). In addition, sustained estrogen treatment improves learning ability, memory, and fine motor skills in animal models (Lacresse and Herndon, 2003). However, results of the Women's Health Initiative show that the use of HRTs can lead to increased risks for heart disease and stroke. There is concern that these estrogens may induce some adverse neurological responses (Shumaker *et al.*, 2003; Espeland *et al.*, 2004). The most well-characterized adverse responses of endogenous and exogenous estrogens are associated with their effects on both breast and endometrial cancer in women (Hilakivi-Clarke, 2000; Bernstein, 2002). There is also concern that fetal or early postnatal exposure of males to estrogens will affect the male reproductive tract functions in adults (Skakkebaek *et al.*, 2001).

### Risks for breast cancer: genetic factors

There are several different risk factors for breast cancer, and there is a strong interplay of genetic and environmental factors. Five to ten percent of all breast cancers are associated with the inheritance of mutations in one of the two major breast cancer susceptibility genes, BRCA1 and BRCA2 (Venkitaraman, 2002). There is an 80% chance of developing breast cancer during a lifetime in women with an inherited BRCA1 or BRCA2 mutation. BRCA1 and BRCA2 are tumor suppressor genes, and their proteins have been implicated in a multitude of different processes, including DNA repair and recombination, cell cycle control, and transcription (Venkitaraman, 2002). Several other genetic mutations also contribute to the risk for breast cancer, including genes such as p53 that also enhance risk for multiple cancers.

### Hormonal risk factors for breast cancer

Several studies have shown that prolonged exposure to the hormone estrogen increases the risk of breast cancer. Epidemiological studies have established a strong link between higher risk of breast cancer and reproductive factors that increase the overall number of menstrual cycles, such as early menarche (before age 12 years), late

menopause (after age 55 years), age of women at first birth (older than 30–35 years), and nulliparity (Russo *et al.*, 1992). Breast cancer risk is lower in women with multiple pregnancies and women with a pregnancy prior to age 24 years (Lambe *et al.*, 1996). Women who are older than 30–35 years of age at first birth are at higher risk compared to nulliparous women (Albrektsen *et al.*, 1994; Rosner *et al.*, 1994). The protective effects of pregnancy against breast cancer are explained by the induction of complete differentiation of the breast that may markedly reduce the carcinogen susceptibility of the fully differentiated mammary gland due, at least in part, to the decreased proliferative activity of parous epithelium (Russo *et al.*, 2000). Studies have shown that the use of HRTs and oral contraceptives for long time periods can also increase the risk of developing breast cancer (Althuis *et al.*, 2003). In some studies, women exposed to diethylstilbestrol (DES) were also found to be at slightly increased risk for breast cancer (Titus-Ernstoff *et al.*, 2001).

### Lifestyle and dietary risk factors

Ionizing radiation is the most well-characterized environmental risk factor for breast cancer. Radiation-induced breast cancer risk depends on various factors, including age at exposure (highest before age 30 years), the status of hormone levels, parity, and other genetic disorders (Coyle, 2004). Other risk factors include solar radiation, light, and chemicals. Solar radiation creates an active form of vitamin D that may lower the risk of breast cancer, and studies show that women who work at night are at higher breast cancer risk. This may be due to decreased vitamin D synthesis and suppression of normal nocturnal production of melatonin by the pineal gland, which increases estrogen release by the ovaries, thereby disrupting circadian patterns (Davis *et al.*, 2001; Schernhammer *et al.*, 2001).

Lifestyle factors such as diet, exercise, smoking, and alcohol consumption are related to an increased risk of developing breast cancer (Key *et al.*, 2003). Confirmation of the risk of dietary fat intake and breast cancer has not been substantiated in large epidemiology studies (Velie *et al.*, 2000; Smith-Warner *et al.*, 2001); however, a dietary pattern of high fiber and low fat intakes is associated with a lower risk of breast cancer in postmenopausal women (Mattisson *et al.*, 2004; Saadatian-Elahi *et al.*, 2004). In some studies, protective effects of some vegetable fats, vitamin E, selenium, and other antioxidants have been observed (Gerber *et al.*, 2003; Gaudet *et al.*, 2004). Decreased ovarian hormone levels decrease the risk of breast cancer in populations in Asia, and this is related to their high consumption of soya products containing significant amounts of the isoflavones

daidzein and genistein, which act as weak estrogens (Mezzetti *et al.*, 1998; Lu *et al.*, 2000).

## ESTROGENS AS RISK FACTORS FOR MALE REPRODUCTIVE TRACT PROBLEMS

Although estrogens are female steroid hormones and influence normal physiology and disease in women, there has been increasing concern regarding the potential adverse effect of estrogenic compounds on the development of the male reproductive tract (Sharpe and Skakkebaek, 1993). Support for the hypothesis that "environmental estrogens" (xenoestrogens) and other endocrine disrupting compounds are responsible for an increase in male reproductive tract problems is derived from laboratory animal, wildlife, and human studies. For example, there are numerous reports of feminized fish in river systems, particularly in the United Kingdom, and this has been linked to their exposure to endogenous estrogens (17 $\beta$ -estradiol/estrone), synthetic contraceptives (17-ethinylestradiol), and industrial byproducts such as alkylphenols (Desbrow *et al.*, 1998; Routledge *et al.*, 1998). There is evidence that nonylphenol and alkylphenols may contribute to estrogenization of fish near sewage outflows.

It has also been suggested that the major concern regarding environmental/dietary estrogens is *in utero* or early postnatal exposure during the important initial periods of male reproductive tract development (Skakkebaek *et al.*, 2001). The potent estrogenic drug DES was extensively used by pregnant women with disastrous consequences for their male and female offspring (Giusti *et al.*, 1995). Females exposed *in utero* developed a high incidence of a rare vaginal adenocarcinoma, whereas male offspring developed a range of responses including deformed genitalia. The effects of DES form one of the bases of the endocrine disruption hypothesis and the opinion that a testicular dysgenesis syndrome that includes low sperm counts, increased hypospadias and cryptorchidism, and testicular cancer may be linked to *in utero* exposure to estrogenic compounds and other endocrine disrupting compounds (Skakkebaek *et al.*, 2001). This opinion and hypothesis have been challenged (Safe, 2000; Handelsman, 2001). However, it has become increasingly clear that humans are exposed to a wide range of xenoestrogens and phytoestrogens, which constitute the major subclass of endocrine disruptors of concern. The following section describes several different structural classes of synthetic estrogenic compounds, phytoestrogens, and estrogens/antiestrogens such as DES

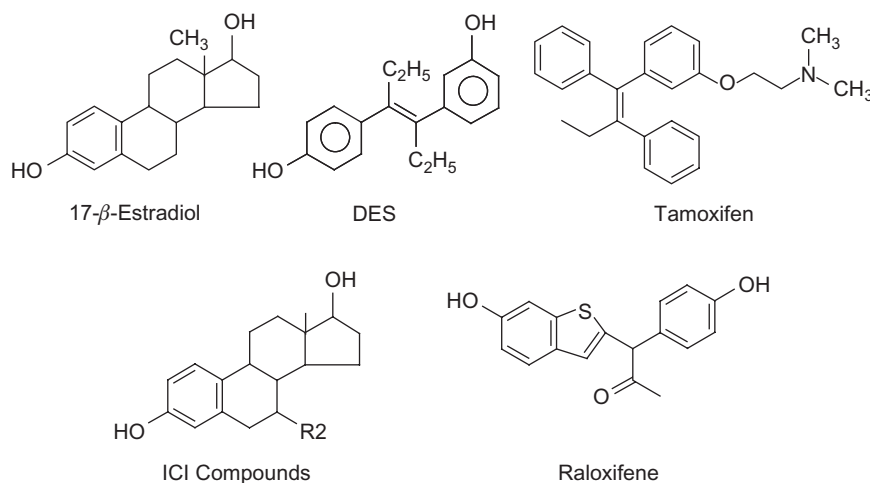
and tamoxifen that have been used as pharmaceutical agents. In addition, the mechanism of estrogen action and implications for risk assessment of these compounds are also discussed.

## ESTROGENIC CHEMICALS OF CONCERN

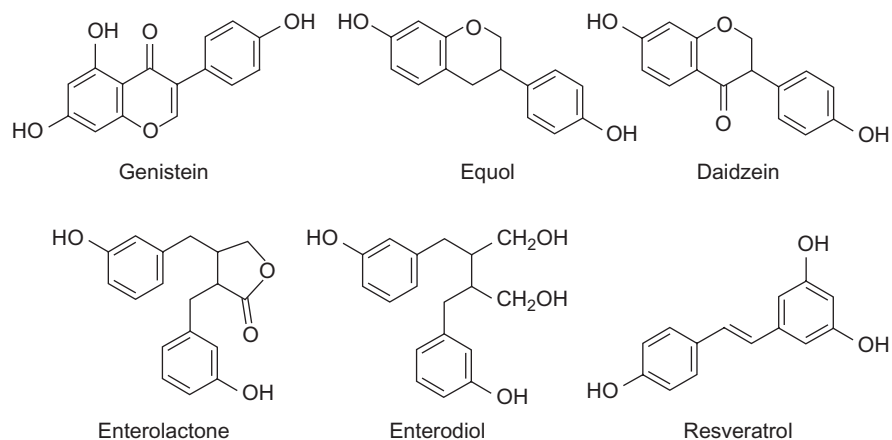
The hypothesis that environmental/dietary estrogens may play a role in male reproductive tract problems and the increased incidence of breast cancer is controversial, and the significance of these compounds on human health is not resolved. Nevertheless, this resulted in legislation in the United States requiring the U.S. Environmental Protection Agency to develop testing procedures for examining industrial compounds for their activity as estrogens/antiestrogens, androgens/antiandrogens, and thyroid hormone mimics. Initial screening studies for estrogens used the MCF-7 cell proliferation assay (E-screen) (Sonnenschein and Soto, 1998), and this was complemented by development of receptor binding and transactivation studies in various cell lines. All of these assays have advantages and disadvantages and for the most part give complementary results on the estrogenic activity of individual compounds.

Steroidal and nonsteroidal estrogens and antiestrogens have been developed as pharmacologic agents, and Figure 75.1 illustrates the structures of 17 $\beta$ -estradiol, the endogenous hormone, DES, and three clinically used antiestrogens – tamoxifen, ICI 182780 (fulvestrant), and raloxifene (Jordan, 2003a,b). It is well known that phytoestrogenic compounds in the diet also constitute a major source of exposure to estrogens. These compounds are present in fruits, nuts, and vegetables and are particularly high in many soya products that are enriched in isoflavonoids (Havsteen, 2002). Figure 75.2 shows the structures of genistein, a major isoflavonoid in soy; equol, a genistein metabolite; and naringenin, a flavonoid that is found in grapefruit. Genistein and equol can be detected in human serum and urine along with the estrogenic lignans enterodiol and enterolactone, and these compounds are used as biomarkers of exposure to dietary phytoestrogenic compounds. Resveratrol is an estrogenic polyphenolic stilbene analog found in grapes and wine.

The list of synthetic estrogenic compounds continues to grow as testing of current and new synthetic industrial chemicals progresses and expands. A major class of xenoestrogens of concern are the alkyl phenols such as nonylphenol, which are widely used in industry as ethoxylates (Soto *et al.*, 1991). These compounds are surfactants and are used in detergents, paints, herbicide/

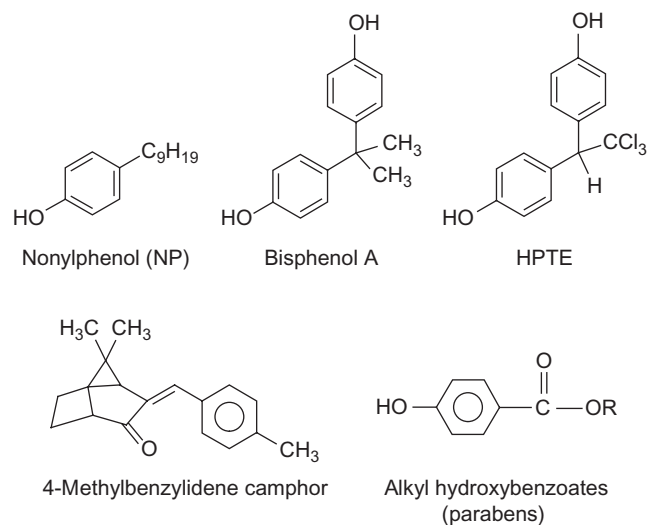


**FIGURE 75.1** Structures of 17β-estradiol, DES, and the clinically used SERMs tamoxifen, ICI compounds (R = C<sub>14</sub>H<sub>24</sub>F<sub>5</sub>OS, fulvestrant), and raloxifene.

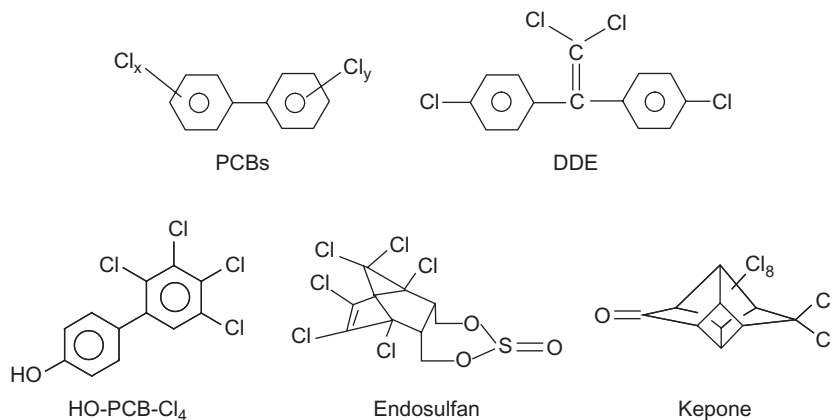


**FIGURE 75.2** Structures of the phytoestrogenic flavonoids (genistein, equol, and daidzein), lignans (enterolactone and enterodiol), and resveratrol.

pesticide formulation, and plastics. Alkyl hydroxybenzoates (parabens) (Figure 75.3), which are used as preservative agents, and cosmetic ultraviolet filters such as 4-methylbenzylidene camphor, which are used in home care products, also exhibit estrogenic activity (Schlumpf *et al.*, 2004; Schreurs *et al.*, 2005). Bisphenol A (BPA) is perhaps one of the most controversial xenoestrogens because it is extensively used in plastic production and plastic lines in which trace levels of BPA have leached directly into food products. This estrogenic compound has also been identified in the environment. Other estrogens of concern include polychlorinated biphenyls (PCBs) and hydroxy-PCBs, which have been identified in the environment and in humans; the pesticide metabolite 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene (DDE); and kepone. These compounds are persistent environmental contaminants, and kepone induced estrogenic poisoning in a group of exposed production workers. The



**FIGURE 75.3** Structures of the aromatic xenoestrogens nonylphenol, bisphenol A, HPTE, 4-methylbenzylidene camphor, and parabens.



**FIGURE 75.4** Structures of the organochlorine xenoestrogens PCBs, DDE, hydroxyl-PCB, endosulfan, and kepone.

compounds illustrated in [Figure 75.4](#) exhibit relatively weak binding to the ER and induce reporter gene expression in transactivation assays in cell culture systems, and some of these compounds exhibit uterotrophic effects in female rodents. The important toxicological question concerning these chemical estrogens is whether they differ only in potency, which is governed by their intrinsic estrogenicity and bioavailability based on their metabolism and pharmacokinetics. On the other hand, because the estrogenic activity of these compounds is receptor dependent (ER), is the estrogenic activity of structurally diverse xenoestrogens more unique and not necessarily governed by intrinsic ER binding affinities and pharmacodynamic factors?

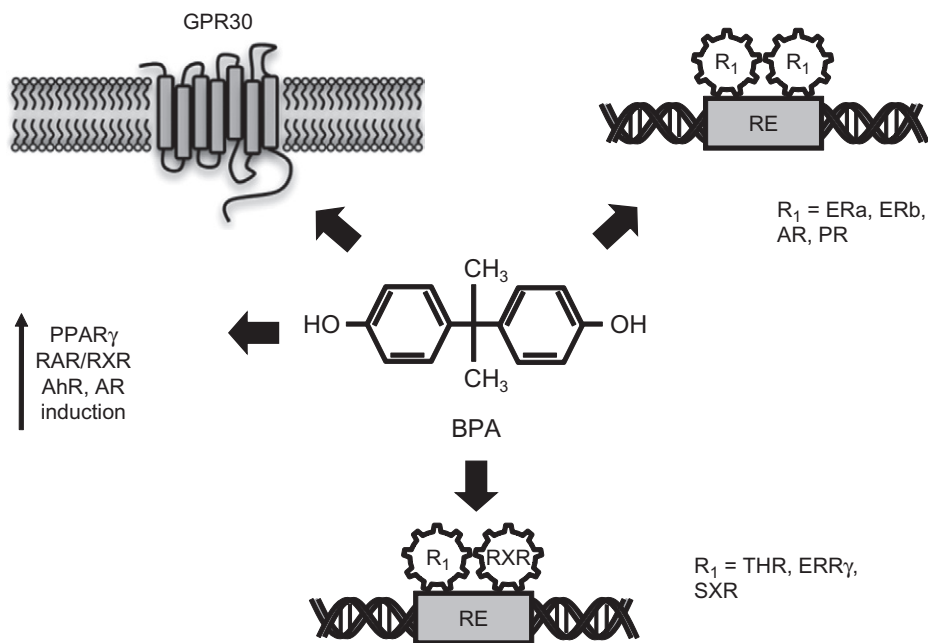
### Case study: bisphenol A

BPA has been the most widely investigated environmental estrogen, and there has been considerable regulatory, scientific, and public concern regarding the use, exposure, and potential adverse effects of this compound. BPA has been a widely used industrial compound with a major application in the production of resins and polycarbonate-based plastic ([Dekant and Volkel, 2008](#)). BPA was initially identified as an estrogenic material leaching out from plastic material, and subsequent studies have identified BPA in foods, environmental samples, and humans ([Dekant and Volkel, 2008](#)). For example, BPA was identified in laboratory plastic products that resulted in some leaching of BPA ([Krishnan \*et al.\*, 1993](#)), and a major source of human exposure to BPA is due to plastic containers of liners that are in contact with food products. The use of BPA in dental sealants also results in human exposure ([Olea \*et al.\*, 1996](#)), and based on extensive sampling, urinary levels of BPA/conjugates are 1–3  $\mu\text{g}/\text{L}$  with an estimated daily uptake of less than 6  $\mu\text{g}$  (<0.1  $\mu\text{g}/\text{kg}/\text{day}$ ) ([Calafat \*et al.\*, 2008](#); [Dekant and Volkel, 2008](#)).

The initial concerns regarding BPA were linked to its estrogenic activity and the potential adverse effects associated with *in utero* exposure because *in utero* exposure to the potent estrogenic drug DES can lead to serious reproductive tract problems in offspring ([Giusti \*et al.\*, 1995](#)). In contrast to DES, BPA is a relatively weak estrogenic compound in terms of activating nuclear ER $\alpha$ , and concentrations in the micromolar range are required for activity ([Gould \*et al.\*, 1998](#); [Yoon \*et al.\*, 2000, 2001](#)). The effects of BPA in the female rat uterus were also consistent with low estrogenic activity, and it was also reported that BPA acted as a partial antiestrogen ([Gould \*et al.\*, 1998](#)). In contrast to the “weak” estrogenic activity observed for BPA in these studies, several reports show that BPA concentrations in the picomolar range activate nongenomic estrogen-dependent signaling pathways in multiple cell types and the potency of BPA is similar to that observed for E2 ([Quesada \*et al.\*, 2002](#); [Wozniak \*et al.\*, 2005](#); [Wetherill \*et al.\*, 2007](#); [Watson \*et al.\*, 2010](#); [Jeng and Watson, 2011](#)).

A search for the word “BPA” on PubMed gives more than 6000 citations, and many of these deal with a myriad of *in vitro* and *in vivo* studies, indicating diverse tissue-/species- and dose regimen-dependent responses that include modulation of pharmacologic/biochemical responses and a wide spectrum of activities ([Figure 75.5](#)). It is also possible that these responses are both estrogen dependent and independent. A major concern regarding BPA is associated with the effects of *in utero* exposure to low levels of BPA on the development of the male and female reproductive tract ([Vom Saal \*et al.\*, 2007](#); [Chapin \*et al.\*, 2008](#); [Allard and Colaiácovo, 2011](#)). Ryan and co-workers (2010a) compared the effects of *in utero* and lactational exposure of BPA and the potent estrogen ethinylestradiol (EE2) on sexually dimorphic behavior, puberty, fertility, and anatomy of female LE rats. EE2 is an oral contraceptive that has been identified in the environment and may contribute to estrogenization





**FIGURE 75.5** BPA exhibits tissue-specific agonist or antagonist activities for multiple receptors and also induces expression of some receptors.

of male fish in contaminated rivers (Jobling *et al.*, 1998, 2006). *In utero* administration of EE2 (0.05–50 µg/kg/day) induced a wide range of dose-dependent reproductive tract disorders and neurobehavioral deficits in the female rats, with only relatively minor effects in males. In contrast, *in utero* exposure to BPA (2, 20, and 200 µg/kg/day) had minimal to nondetectable effects on female and male offspring (Ryan *et al.*, 2010a), and these results were consistent with those of previous reports (Cagen *et al.*, 1999; Ema *et al.*, 2001; Tinwell *et al.*, 2002; Tyl *et al.*, 2002). Based on the results reported by Ryan and co-workers, it was suggested that future BPA research funding “looks increasingly like an investment with a nil return” (Sharpe, 2010).

Another concern regarding the potential adverse effects of BPA and environmental estrogens is their potential role in the obesity epidemic, particularly in North America, and the increase in multiple diseases associated with obesity (e.g., diabetes) (Newbold *et al.*, 2005, 2007, 2009). The concern about this response is derived, in part, by reports showing that *in utero* exposure to DES results in obesity in adult mice (Newbold *et al.*, 2005, 2007) and by studies showing that early life exposure of rodents to BPA increases body weights in adult offspring (Rubin *et al.*, 2001; Akingbemi *et al.*, 2004; Miyawaki *et al.*, 2007). A review of the data by one panel of scientists suggested that a role for BPA in obesity is unclear (Chapin *et al.*, 2008), whereas another panel of experts expressed a higher level of concern regarding the contribution of BPA to a large number of diseases

(Vom Saal *et al.*, 2007). A study investigated the effects of relevant doses of BPA (0.25 µg/kg/day) and DES (1 µg/kg/day), and the results showed a faster rate of early growth in the BPA-exposed mice compared to controls. However, the authors reported that these differences were no longer apparent when the mice reached adulthood, even when they were tested on a high-fat diet (Ryan *et al.*, 2010b). A commentary on this study concluded that it is unlikely that BPA exposure in early life in humans is a factor in predisposing to metabolic syndrome disorders in adulthood (Sharpe and Drake, 2010). Nevertheless, the controversy regarding the effects of BPA on various laboratory animal models remains a contentious issue among scientists.

Human studies correlating background BPA levels with disease have been limited. Braun and co-workers (2009) estimated *in utero* exposures from maternal urine samples collected at two times (16 and 26 weeks) during gestation, and there was an association between 16-week BPA concentrations and externalizing scores among the 2-year-old offspring. Refinements to this approach and more prolonged observation of the offspring should provide important answers in the future. Two related studies used urinary BPA levels in participants in the National Health and Nutrition and Examination Study (NHANES) to investigate correlations between BPA and diseases (Lang *et al.*, 2008; Braun *et al.*, 2009). Cardiovascular disease, diabetes, and enhanced liver enzymes were associated with increased urinary BPA levels, although the diabetes correlation was variable

at the two sampling time points (2003–2004 and 2005–2006). This type of approach coupled with case–control and prospective studies will also be useful in assessing possible linkages between BPA and human diseases.

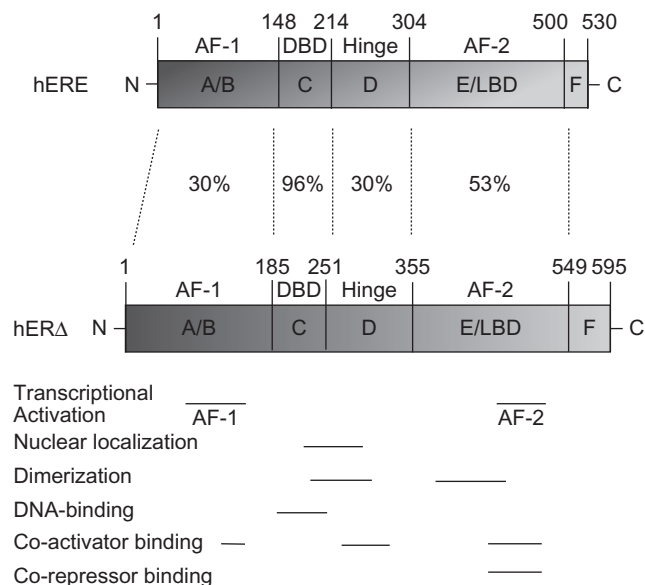
## MECHANISMS OF ESTROGEN-INDUCED TRANSACTIVATION

### ER $\alpha$ and ER $\beta$ domain structure

The ER is a member of the nuclear receptor superfamily of transcription factors that exhibit modular structures and similar mechanisms of action. For example, the transactivation function of ER is mediated by two separate but not mutually exclusive transactivation domains, namely ligand-independent activation function (AF)-1 (within the A/B domain) and ligand-dependent AF-2 (within the ligand binding domain (LBD)) (Tora *et al.*, 1989) (Figure 75.6). The A/B domain is the least conserved region between ER $\alpha$  and ER $\beta$ , with only 30% similarity at the amino acid level. The activity of AF-1 in ER $\beta$  is negligible on estrogen-responsive element (ERE) reporter constructs compared to that of the AF-1 of ER $\alpha$  in several different cell lines (Cowley and Parker, 1999).

ER $\alpha$  and ER $\beta$  also exhibit distinctive responses to the synthetic antiestrogens such as tamoxifen and raloxifene, which act as partial ER agonists for ER $\alpha$  and as pure ER antagonists for ER $\beta$  (Barkhem *et al.*, 1998). The functional differences between the respective A/B regions of ER $\alpha$  and ER $\beta$  may explain their differences in ligand-dependent activation (Matthews and Gustafsson, 2003). The AF-1 region of ER $\alpha$  interacts with different transcriptional regulators and co-activators that affect ligand-independent transactivation. The activity of AF-1 is also regulated through kinase-dependent phosphorylation, and the individual pathways involved vary with cell and promoter context (Tzukerman *et al.*, 1994). In most cell lines, both AFs act synergistically to attain maximum transcriptional activity, whereas in other cells only one AF may be activated (Benecke *et al.*, 2000).

The DNA binding domain (DBD; region C) is highly conserved between ER $\alpha$  and ER $\beta$  and exhibits 96% identity. This domain contains two zinc fingers (CI and CII), as observed for other nuclear receptors. The DBDs of both ER $\alpha$  and ER $\beta$  bind with high affinity to EREs. The C-terminal E/F region encompasses the LBD, a co-regulator binding surface, a dimerization domain, another nuclear localization signal, and AF-2 (Nilsson *et al.*, 2001). Significant homology between the two receptors exists in the E/F region, and both proteins display essentially the same binding affinity for E2 and many other estrogenic compounds (Kuiper *et al.*, 1997). However, the



**FIGURE 75.6** Structural domains of human ER $\alpha$  and ER $\beta$ . The percentage identity between the individual domains at the amino acid level is indicated. (Modified from Pearce and Jordan (2004) and Koehler *et al.* (2005).)

two receptors differ in their binding affinities for only a few ligands, including antiestrogens and phytoestrogens. For example, the phytoestrogen genistein binds with approximately a 30-fold higher affinity for ER $\beta$  than for ER $\alpha$  (Barkhem *et al.*, 1998).

## XENOESTROGENS AND PHYTOESTROGENS AS SELECTIVE ER MODULATORS

### Complexity of estrogenic activity

Results of X-ray crystallographic analysis clearly demonstrate that both ER agonists differentially bind the ER and induce compound-specific changes in the bound complex. Although tamoxifen and E2 induce distinct conformations of the ER and exhibit antiestrogenic and estrogenic activity in breast cancer, these compounds both induce ER-dependent activity in the uterus, and prolonged treatment with tamoxifen increases the risk for endometrial cancer (Bernstein, 2002). Tamoxifen is also an ER agonist in the bone and vascular system, and there are tissue- and species-specific ER agonist or antagonist activities in animal models: tamoxifen is an ER antagonist in chicks, a partial ER agonist/antagonist in rats, and an ER agonist in several short-term assays in mice (MacGregor and Jordan, 1998). Studies on tamoxifen and other ER agonists have led to the

**TABLE 75.1** The SERMs tamoxifen, raloxifene, ICI 164384, and E2 differentially activate ER $\alpha$  and also exhibit unique *in vivo* biologies

SERM	ER $\alpha$				ER $\alpha$ -AF-1				ER $\alpha$ -AF-2			In vivo ER activity		
	A/B	C/D	E	F	A/B	C/D	E***	F	C/D	E	F	Bone	Breast	Uterus
E2		+++ <sup>a</sup>				+++				+++		Ag <sup>b</sup>	Ag	Ag
Tamoxifen		+				+				ND		Ag	Ant	Ag
Raloxifene		ND				+				ND		Ag	Ant	–
ICI 164,384		ND				ND				ND		–	Ant	Ant

From Tzukerman *et al.* (1994).

<sup>a</sup>E2 induced maximal responses in all assays (+++), and responses <40% (+) of that observed for E2 are indicated. ND indicates no significant induction or inhibition of activity.

<sup>b</sup>Ag and Ant indicate ER agonist and antagonist, respectively; – indicates no agonist activity.

development of the acronym selective ER modulators (SERMs), which exhibit a complex pharmacology and induce tissue-specific ER agonist or antagonist activities. These structure-dependent differences are related to differential activation of estrogen-responsive genes/pathways and due to several factors (Katzenellenbogen *et al.*, 1996; Smith and O'Malley, 2004), including

- the structure of the estrogenic compound;
- tissue-specific expression of ER subtype (ER $\alpha$  and ER $\beta$ ) or variant;
- tissue-specific expression of critical co-activators and other co-regulatory proteins; and
- promoter context and chromatin state, which is dependent on histone methylation or acetylation, promoter methylation, and expression of critical modulating *trans*-acting factors.

## Development of bioassays for clinically relevant SERMs

SERMs were primarily developed for treatment of hormone-dependent diseases or conditions, and they define the increasing complexity of ER action. If xenoestrogens and phytoestrogens are SERMs, then their estrogenic and antiestrogenic activities will also be tissue specific and their role in causation or protection from hormone-dependent problems will depend on the structure of the individual compound, the amount of exposure (assuming a threshold), and the time of exposure where critical modifications of hormone-responsiveness are induced. The structurally diverse SERMs – E2, tamoxifen, raloxifene, and ICI 164,384 – have unique *in vivo* biologies and were used as model compounds to develop an *in vitro* bioassay that distinguishes between these compounds (Tzukerman *et al.*, 1994; McDonnell *et al.*, 1995). This bioassay utilizes the modular structure of ER $\alpha$  and ER $\beta$  in which the various domains (E–F) exhibit both separable and overlapping functions that

govern their interactions with other co-regulatory proteins and promoter DNA. AF-1 and AF-2 are located in the A/B and E domains, respectively, and are particularly important for this assay system. Table 75.1 shows that these four compounds differentially induce transactivation in human hepatoma HepG2 cells transfected with the E2-responsive pC3 construct (human complement C3 promoter linked to the luciferase gene) and expression plasmids for wild-type ER $\alpha$ , ER $\alpha$ -AF-2 in which the AF-1 domain has been deleted, or ER $\alpha$ -AF-1 in which the critical amino acids in AF-2 have been mutated (amino acids D538N, E542Q, and D545N). E2 induces transactivation in HepG2 cells transfected with wild-type/variant ER $\alpha$ ; in contrast, raloxifene activates ER $\alpha$ -AF-1, tamoxifen activates ER $\alpha$  and ER $\alpha$ -AF-1, and ICI 164,384 does not induce or inhibit transactivation.

## Xenoestrogens and phytoestrogens as SERMs

This assay has been used for distinguishing between different structural classes of xenoestrogens and phytoestrogens, including BPA (2',4',6'-trichloro-4-biphenylol (Cl<sub>3</sub>-PCB-OH), 2',3',4',5'-tetrachloro-4-biphenylol (Cl<sub>4</sub>-PCB-OH), *p*-*t*-octylphenol (OP), *o*-nonylphenol (NP), naringenin, kepone, resveratrol, and 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). E2, ICI 182,780, and 4-hydroxytamoxifen were used as positive controls (Gould *et al.*, 1998; Yoon *et al.*, 2000, 2001). Results obtained in several cell lines using the E2-responsive complement pC3-luc construct or a construct (pERE<sub>3</sub>) containing three EREs linked to luciferase demonstrate that xenoestrogens/phytoestrogens also differentially induce transactivation.

Table 75.2 compares the maximal induced responses observed for these compounds using human hepatoma HepG2 and human U2 osteogenic cancer cell lines transfected with pERE<sub>3</sub> and ER $\alpha$ , ER $\alpha$ -AF-1, or ER $\alpha$ -AF-2 (Yoon *et al.*, 2001). Even among structurally related compounds such as the hydroxy-PCBs, alkylphenols,

TABLE 75.2 Different structural classes of xenoestrogens, phytoestrogens, E2, and 4-hydroxytamoxifen differentially induce transactivation in HepG2 and U2 cells transfected with pERE<sub>3</sub>, wild-type, and variant forms of ER $\alpha$ 

	ER $\alpha$				ER $\alpha$ -IAF-1				ER $\alpha$ -AF-2		
	A/B	C/D	E	F	A/B	C/D	E***	F	C/D	E	F
	HepG2		U2		HepG2		U2		HepG2		U2
17 $\beta$ -estradiol	+++ <sup>a</sup>		+++		+++		+++		+++		+++
4-Hydroxytamoxifen	+		ND		ND		ND		ND		ND
NP	+		+		++		ND		++		ND
OP	+		+		+		+		ND		ND
HO-PCB-Cl <sub>4</sub>	++		++		+		ND		++		+++
HO-PCB-Cl <sub>3</sub>	++		++		+		++		+++		+++
HPTE	+++		+		+		ND		+++		++
BPA	++		++		+		++		++		+++
Kepone	ND		ND		+		ND		+		ND
Naringenin	ND		ND		ND		ND		ND		ND
Resveratrol	ND		ND		ND		ND		ND		ND

From Yoon *et al.* (2001).

<sup>a</sup>E2 induced a maximal response (+++) in all assays. A significant induction response of >40% (+++) or <40% (+) of that observed for E2 and no significant induction (ND) are indicated. ICI 182,780 gave an ND for all responses. The doses used were 10 nM and 1  $\mu$ M for E2 and 4'-hydroxytamoxifen, respectively, 100  $\mu$ M for BPA, and 10  $\mu$ M for the remaining compounds.

and bisphenolics HPTE and BPA, there were some significant differences in their induction of transactivation. Moreover, using a similar approach in HepG2, U2, and MDA-MB-231 cancer cell lines transfected with a pC3-luc construct, similar differences were observed (Yoon *et al.*, 2000). For example, the two bisphenolic compounds HPTE and BPA exhibit a similar pattern of transactivation in many assays, except that in U2 cells BPA but not HPTE induces transactivation in cells transfected with ER $\alpha$ -AF-1. It is also apparent from other *in vivo* and *in vitro* studies that BPA and HPTE exhibit differences in their estrogenic activities. For example, HPTE was a more potent estrogen than BPA in the female rat uterus; however, in combination with E2, lower doses of BPA inhibited E2-induced uterine progesterone receptor binding and peroxidase activity (Gould *et al.*, 1998). HPTE versus BPA also exhibited other differences in HepG2 cells in which both HPTE and BPA are ER $\alpha$  agonists, whereas HPTE is an ER $\beta$  and androgen receptor (AR) antagonist and BPA is an ER $\beta$  agonist and did not affect AR in this cell line (Gaido *et al.*, 1999). These activities can also vary in other cell contexts but clearly demonstrate significant *in vitro* and *in vivo* biological differences between HPTE and BPA, suggesting that structurally diverse estrogenic compounds are SERMs.

Several other *in vitro* studies have demonstrated significant differences among different structural classes of estrogenic compounds and the planar phytoestrogens coumestrol and genistein. For example, an ER $\alpha$  mutant (D351G) is activated by E2 and DES but not by 4'-hydroxytamoxifen or the nonplanar HPTE and related compound (Jordan *et al.*, 2001). It was also reported that the isoflavones daidzein, biochanin, and genistein are

ER $\beta$ -selective, and this is due to their preferential induction of co-activator interactions with AF-2 of ER $\beta$  compared to AF-2 of ER $\alpha$  (An *et al.*, 2001). Other reports also show that activation of gene expression in *in vitro* assays by structurally diverse xenoestrogens/phytoestrogens depends on the promoter context (i.e., different consensus vs. nonconsensus EREs) and on co-activator interactions (Hall *et al.*, 2002; Mueller *et al.*, 2004). Studies have also demonstrated structure-dependent activation of nongenomic kinase pathways by xenoestrogens/phytoestrogens (Li *et al.*, 2006). Moreover, it has also been shown that some estrogenic compounds activate kinases via ER-independent pathways by directly binding to a G protein-coupled receptor (GPR30) (Revankar *et al.*, 2005; Thomas *et al.*, 2005). The structure dependence on this ER-dependent pathway has yet to be determined. These results clearly indicate that xenoestrogens/phytoestrogens are SERMs.

## XENOESTROGENS AND PHYTOESTROGENS AS SERMS AND IMPLICATIONS FOR RISK ASSESSMENT

Xenoestrogens and phytoestrogens differentially activate wild-type and variant ER $\alpha$  *in vitro*, suggesting that these compounds are SERMs. This implies that their estrogenic activity and potency cannot be determined by simple ER binding or gene expression assays. Thus, to fully understand the estrogenic or antiestrogenic activities of phytoestrogens and xenoestrogens, studies



will have to focus on their tissue-specific impacts at various critical periods of exposure. Risk assessment of these compounds will be complex and dependent on all the variables indicated previously. Moreover, because individual SERMs exhibit unique biologies, the overall impact of mixtures of these compounds may not be additive. Another complication associated with the mechanisms of action and risk assessment of many xenoestrogens is due to their multiple tissue/species activities, and this is illustrated by BPA and its interaction with receptors. As indicated previously, BPA activates ER $\alpha$  and ER $\beta$  (Kuiper *et al.*, 1997) and also binds and activates GPR30 (Thomas and Dong, 2006; Dong *et al.*, 2011). BPA also induces CaBP-9k, a progesterin-responsive gene in the mouse uterus (Jung *et al.*, 2005), and induces neuronal PR mRNA levels in rats (Funabashi *et al.*, 2004). BPA also acts as an AR agonist and antagonist in different assays (Lee *et al.*, 2003) and exhibits thyroid hormone receptor antagonist activity (Moriyama *et al.*, 2002; Zoeller *et al.*, 2005). BPA also binds and/or activates estrogen-related receptor  $\gamma$  (EER $\gamma$ ) (Matsushima *et al.*, 2007; Abad *et al.*, 2008) and the steroid and xenobiotic receptor (SXR) and induces NR4A1 (Nur77) in mouse testicular Leydig cells (Song *et al.*, 2002). Moreover, it has been reported that BPA induces the aryl hydrocarbon receptor (AhR), AR, retinoid receptors, and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), and this can also result in modulation of receptor-mediated responses (Nishizawa *et al.*, 2005a,b; Kruger *et al.*, 2008; Kwintkiewicz *et al.*, 2010). These factors highlight the challenges faced by scientists and regulators in addressing the health risks and benefits of estrogenic compounds.

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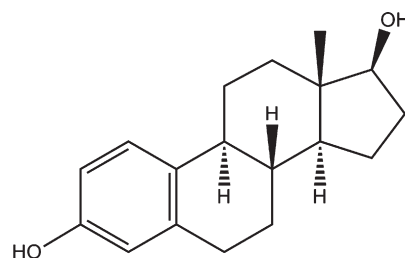


# Phytoestrogens

Michelle Mostrom and Tim J. Evans

## INTRODUCTION

Phytoestrogens are nonsteroidal, natural plant compounds that are structurally or functionally similar to mammalian estrogens, particularly 17 $\beta$ -estradiol (Figure 76.1). Typically, phytoestrogens or their active metabolites exert their estrogenic effect on the central nervous system and on the reproductive system of males and females, inducing estrus and stimulating growth of the genital tract and mammary glands in females. The classic test for estrogenicity of compounds is proliferation of the female reproductive tract. Phytoestrogens may bind to estrogen receptors, mimicking the conformational structure of estradiol (Kuiper *et al.*, 1997, 1998), and act as agonists, partial agonists, or antagonists inducing estrogen-responsive gene products and may exert metabolic effects not related to estrogen receptors. Phytoestrogens are considered endocrine disruptors because these chemicals may interfere with the body's endocrine system and produce adverse developmental, reproductive, and neurological effects in both humans and animals. A large volume of literature has been published on phytoestrogens with regard to their beneficial effects in humans related to reducing atherosclerosis, osteoporosis, angiogenesis, diabetes, and vasomotor effects (hot flushes) at menopause and acting as antioxidants, antineoplastics, anti-inflammatories, and probiotics. Fewer publications discuss adverse effects of phytoestrogens causing infertility in livestock and possible impaired reproductive processes in humans. This chapter focuses on the most extensively studied phytoestrogens found in legumes and beans, such as the isoflavones and coumestans, which may affect reproduction.



17BETA-ESTRADIOL

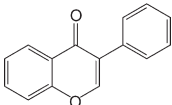
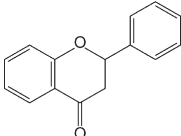
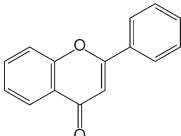
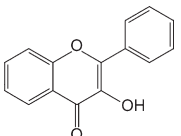
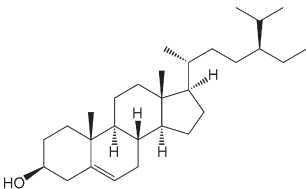
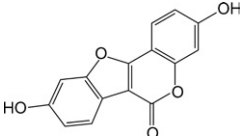
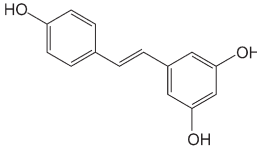
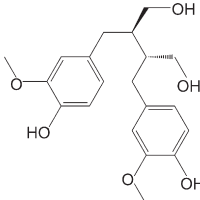
(8*R*,9*S*,13*S*,14*S*,17*S*)-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6*H*-cyclopenta[*a*]phenanthrene-3,17-diol

FIGURE 76.1 Chemical structure of the estrogen found in animals or 17 $\beta$ -estradiol.

## Description

Phytoestrogens are polyphenolic compounds that can be divided into several broad categories (Table 76.1). Many phytoestrogens are grouped into flavonoids and isoflavonoids, including the isoflavone compounds found in soybeans (*Glycine max* L.), red clover (*Trifolium pretense* L.), and white clover (*Trifolium repens* L.). These compounds include daidzein, genistein, formononetin, biochanin A, and glycitein. The normal isoflavone concentration in red clover is between 0.5 and 2.5% of dry matter, which can be 2–10 times greater than the isoflavone concentration found in soybeans. Hops and beer may contain a very potent phytoestrogen, the flavanone 8-prenylnaringenin.  $\beta$ -Sitosterol is one of several plant sterols widely distributed in the plant kingdom, with a chemical structure similar to that of cholesterol. It is

TABLE 76.1 Categories of phytoestrogens and selected compounds

Category	Phytoestrogen examples	Dietary sources	Basic chemical structure
Isoflavones	Daidzein Genistein Fomononetin Biochanin A Glycitein	Clovers (red and white) Soybeans Beans Split peas	 ISOFLAVONE
Flavanones	Naringenin	Hops (8-prenylnaringenin) Apples, red onions	 FLAVANONE
Flavonoids	Apigenin Luteolin	Parsley Capsicum pepper Alfalfa	 FLAVONE
	Quercetin Kaempferol	Tomatoes Broccoli Apples Onions	 FLAVONOL
Plant sterols	B-sitosterol	Corn Soybeans Sugar beet forage Saw palmetto ( <i>Serenoa repens</i> ) Avocados Pistachios and almonds Wood	 BETA-SITOSTEROL
Coumestans	Coumestrol	Legumes (alfalfa, clover) Spinach Split peas, lima beans Soybean sprouts	 COUMESTROL
Stilbenes	<i>Trans</i> -resveratrol ( <i>trans</i> -3,5,4'-trihydroxystilbene)	Grape skin (red wine) Peanuts	 Resveratrol
Lignans	Secoisolariciresinol, matairesinol	Flaxseed (linseed) Squash, pumpkin seeds Tea (black and green) Sunflower seeds Strawberries Cranberries Brans	 Secoisolariciresinol

Adapted from Patisaul and Jefferson (2010).

found in corn, soybeans, sugar beets, avocados, pistachios, pecans, almonds, and saw palmetto. Coumestrol is a potent estrogenic phytoestrogen in the coumestan group. Coumestrol may be found in alfalfa (*Medicago sativa* L.), white clover, spinach, and soybean sprouts. The stilbenes, such as *trans*-resveratrol, are found in red wine (grape skin) and peanuts. Lignans are compounds found in plant cell walls and fiber-rich foods, seeds (flax and sesame seeds), berries, cereals, nuts, and fruits. Typically, a mixture of phytoestrogens can be found in plants and processed food. An excellent database for foods and flavanoid contents can be found online at the U.S. Department of Agriculture ([www.ars.usda.gov](http://www.ars.usda.gov)). In addition to phytoestrogen exposure through plant products, the environmental distribution of phytoestrogens in streams in the United States has been evaluated (Kolpin *et al.*, 2010). Phytoestrogens (particularly formononetin) were commonly detected in several rivers and streams throughout Iowa, but the concentrations were generally less than 50 ng/L and not considered to contribute to an estrogenic effect, although the impact of long-term, low-level exposures of contaminants in streams is unknown.

This chapter does not include the estrogenic *Fusarium* spp. mycotoxins, zearalenone, zearalanols, and zeaxenols, which are resorcylic acid lactones produced as secondary fungal metabolites in plants and grasses. Additional information on phytoestrogens can be found elsewhere with regard to mechanistic effects (Rosselli *et al.*, 2000; Bhavnani *et al.*, 2008), physiology (Kurzer and Xu, 1997; Tham *et al.*, 1998; Benassayag *et al.*, 2002; Patisaul and Jefferson, 2010; Pilšáková *et al.*, 2010), and reproductive functions (Whitten and Patisaul, 2001; Dusza *et al.*, 2006; Cederroth *et al.*, 2009; Baber, 2010).

## HISTORICAL BACKGROUND

More than 50 years ago in Australia, a syndrome of temporary or permanent infertility occurred in female sheep grazing subterranean clover (*Trifolium subterraneum* L.) containing high concentrations of isoflavone phytoestrogens, particularly formononetin (Adams, 1995). Temporary infertility was related to direct effects of phytoestrogen on the ovarian follicle decreasing ovulation and increasing embryo mortality and was associated with abnormalities of ovum transport and uterine function. Prolonged exposure to growing, green subterranean clover caused permanent infertility in ewes that was associated with morphological changes in the cervix, including thick, fused cervical folds and the appearance of cystic tubular glands. Changes in the uterus included development of cystic uterine glands and

mild endometritis. The cervical mucus became watery and lost viscoelasticity, allowing loss of spermatozoa from the cervix and reducing the chances for conception. External genitalia of some ewes underwent masculinization, with fusion of the vulvar lips at the lower commissure and hypertrophy of the clitoris. The permanent changes in cervical structure were analogous to the organizational effects of estrogen reported in mice treated with estrogen neonatally and in women exposed to diethylstilbestrol during fetal development. Unlike sheep, cattle apparently are not permanently affected by phytoestrogens. However, elevated concentrations of phytoestrogens can negatively impact cattle reproduction and induce estrogenic clinical signs. For example, concentrations of coumestrol greater than 25–30 ppm in a ration (on a dry weight basis), or even lower coumestrol concentrations when feedstuffs are fed at high proportions of a ration to dairy cows, can cause ovarian dysfunction, early embryonic death, and repeat breeding in cattle. The potential health benefits and adverse effects of phytoestrogens in humans and animals related to reproduction are described in this chapter.

## PHARMACOKINETICS/ TOXICOKINETICS

### Plant impact

The concentration of phytoestrogens in plant material varies widely. In addition to participating in plant defense, these compounds play a role in attraction of pollinators and seed-dispersing organisms. Phytoestrogens are not translocated within the vascular pathways of a plant but are synthesized and degraded in localized areas that vary with the specific tissue. For example, coumestrol concentrations are found in higher concentrations near the top segment of the alfalfa plant canopy compared to the lower part of the plant (Seguin *et al.*, 2004).

A number of factors affect production of phytoestrogens. Plant fungal infections, animal predation, or insect invasions may increase phytoestrogen production. Growing conditions, particularly temperature and rainfall, can dramatically affect phytoestrogen concentrations in legumes. In cool, wet spring and fall conditions, legumes may contain high concentrations of phytoestrogens (isoflavones and coumestrol). Generally, the concentrations of phytoestrogens decrease with successive cuttings of legumes in a season, with coumestrol at higher concentrations in early and late maturity of the alfalfa crop. Seguin and Zheng (2006) reported that coumestrol concentrations in alfalfa were lower in harvests

**TABLE 76.2** Typical phytoestrogen concentrations in plants used for livestock forage and soy food

Plant	Phytoestrogen	Concentration (mg/kg or ppm dry weight)
Alfalfa	Coumestrol	25–65 <sup>a</sup>
Red clover	Formononetin and biochanin A	3,000–15,000 <sup>b</sup>
	Genistein	300–1,500
	Daidzein	<300
White clover	Isoflavonoids	100–600
	Coumestrol	<10
Soy foods	Daidzein and genistein	~1,000–3,500

From Saloniemi *et al.* (1995) and Franke *et al.* (1995).

<sup>a</sup>Concentrations from 18 to >180 mg/kg coumestrol have been associated with infertility in cattle (Mostrom, 2010).

<sup>b</sup>Concentrations >500 to 750 mg/kg have been associated with infertility in cattle (Mostrom, 2010).

of first-year seeding than in harvests of the postseeding years. The variety of alfalfa cultivar had little impact on coumestrol concentrations. Coumestrol may also be found in alfalfa cubes, extracts, and powders, which can be incorporated into nutraceuticals or livestock and pet feeds.

Fresh herbage tends to have higher concentrations of phytoestrogens than silage or hay (Sivesind and Seguin, 2005). Sivesind and Seguin reported that the red clover cultivar “Start” was consistently lower in detectable isoflavones during multiyear and multisite trials. Lundh (1995) estimated the daily consumption of phytoestrogens by dairy cows on red clover forage to be 50–100 g. Table 76.2 lists typical concentrations of phytoestrogens in legume feeds and soy foods.

In soy foods, boiling, milling, or processing of the commodity does not appear to destroy daidzein or genistein, but roasting soybeans can reduce these isoflavones by 15% (Franke *et al.*, 1995). Three estrogenic isoflavones – daidzein, genistein, and glycitein – were found in soy foods in four chemical forms: aglycone, glucoside, acetylglucoside, and malonylglucoside. Fermentation and processing of soy foods increased the aglycone and glucoside forms of the isoflavone, respectively. Soy processing appears to influence isoflavone bioavailability; the unconjugated isoflavones in fermented soy food may be more bioavailable than glucosides. The total isoflavone content of raw soy beans ranges widely from 18 to greater than 500 mg/100 g.

## Animal biotransformation

Similar to any drug or toxin, the dose or intake of phytoestrogens is not equivalent to the dose at the active site or receptor in tissue. Dietary phytoestrogens undergo

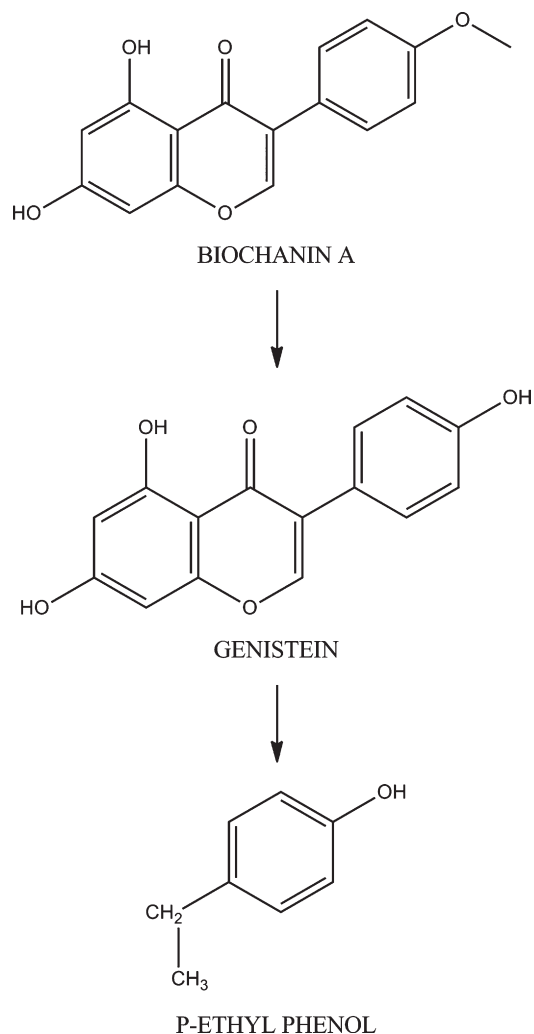
the processes of absorption in the gastrointestinal tract, biotransformation, distribution, and excretion in urine, bile, feces, and milk. Effects of phytoestrogens may vary with the individual phytoestrogen; species exposed; sex; the route, dose, and duration of exposure; and the timing of exposure during reproductive development and cycling. Most phytoestrogens occur in plants as biologically inactive glycoside conjugates with glucose or carbohydrate moieties. Plant glycosides can be hydrolyzed by plant enzymes, or after consumption the glycosides are hydrolyzed and further demethylated in the acidic gut or rumen by microbes and the heterocyclic phenols (aglycones) are free in the gastrointestinal tract. Gut flora may become adapted over a matter of days to the diet and expand their populations for enhanced metabolism. Microbial metabolism of isoflavones can vary greatly between individuals. Isoflavone absorption and bioavailability in humans can vary with intestinal microbial population, gut transit time, fecal digestion rates, and fiber content in the diet (Neilsen and Williamson, 2007).

## Absorption

In ruminants, a majority of the metabolic transformations of phytoestrogens occur in the rumen by microbial action. Complete metabolic pathways have not been defined (Lundh, 1995). Basically, in ruminants, biochanin A is demethylated to genistein and via ring cleavage to *para*-ethyl phenol and organic acids (Figure 76.2). *Para*-ethyl phenol is considered a non-estrogenic compound. Formononetin is primarily demethylated to daidzein and further metabolized via hydrogenation and ring fission to equol (Figure 76.3). Equol contains a chiral center and occurs as two distinct diastereoisomers with R-(+)-equol and S-(–)-equol; intestinal bacteria synthesize exclusively the S-(–)-equol enantiomer that has selective affinity for the estrogen receptor- $\beta$  (Setchell and Clerici, 2010a,b). Formononetin can also undergo reduction to *O*-methyl equol or can be metabolized to *O*-desmethylangolensin. In ruminants, daily consumption of phytoestrogens in the diet can lead to adaption and a larger population of rumen microbes capable of phytoestrogen metabolism. Therefore, estrogenic activity of biochanin A and genistein in ruminants is generally limited to a few initial days of exposure when the unadapted rumen microbes have slower metabolism to non-estrogenic metabolites *para*-ethylphenol and phenolic acid. With the ingestion of formononetin and daidzein, metabolism may lead to compounds with less or greater estrogenic activity (i.e., equol).

Enterodiol and enterolactone are the active, estrogenic mammalian lignans formed by microbes in the intestinal tract from plant lignans matairesinol and secoisolariciresinol and their glycosides (Wang, 2002) (Figure 76.4).





**FIGURE 76.2** Schematic of rumen metabolism of biochanin A to genistein (Cox and Davies, 1988).

Only the unconjugated forms (aglycones) and active metabolites appear to exert estrogen-like activity in animals. Most hydrolyzed phytoestrogens are conjugated by glucuronic acid (a minor fraction is conjugated with sulfate) in the gut epithelium, which is a major mechanism for detoxification of phytoestrogens. A small portion of the free, hydrolyzed compounds are absorbed through the gut or rumen mucosa and reach the blood circulation unconjugated. Absorption of phytoestrogens is fairly rapid. In cattle, formononetin and daidzein (free and conjugated) reached a maximum level in plasma within 1 h after feeding (Lundh, 1995). The unconjugated phytoestrogens reaching the circulation are conjugated by liver (hepatic UDP-glucuronosyltransferases and sulfotransferases) and other tissues, including kidney. Glucuronide conjugated compounds and free phytoestrogens circulate throughout the body. Mammalian lignans and isoflavones can be detected in serum, bile,

and urine following phytoestrogen consumption. Like endogenous estrogens, these conjugated phytoestrogens undergo enterohepatic circulation. The metabolism of coumestrol has not been characterized.

Conjugated equol in the plasma of cattle or sheep is approximately 95–99% of total equol, whereas in pigs approximately 50–70% of total equol is conjugated (Lundh, 1995). The estrogenic effects are related to free compounds and active metabolites, such as equol, which is suggested to have 0.061% of the potency compared to 17 $\beta$ -estradiol (Markiewicz *et al.*, 1993). Equol was considered the primary chemical responsible for infertility in sheep consuming isoflavones in subterranean clover. Ingestion of high concentrations of red clover silage by ruminants can lead to extremely high concentrations of unconjugated equol in plasma and potency 100 times higher than the 17 $\beta$ -estradiol activity during estrus. Following consumption of mixed red clover–grass silage, the concentration of free equol was approximately 10 times greater in bovine plasma compared with ovine plasma, suggesting that the difference in isoflavone sensitivity between cattle and sheep was not caused by differences in metabolism or detoxification of formononetin and daidzein (Lundh *et al.*, 1990).

### Tissue distribution

Data are limited on the tissue distribution of phytoestrogens. Following an intravenous injection of daidzein in rats (40mg/kg body weight), daidzein was detected at high concentrations in plasma, liver, lung, and kidney and at lower concentrations in spleen, heart, and skeletal muscle (Yueh and Chu, 1977). Tissue distribution of isoflavones was determined in two lactating ewes after being fed red clover silage for 1 month (Urpí-Sarda *et al.*, 2008). The fermented silage contained only aglycones and provided a daily intake of approximately 157mg/kg body weight of isoflavones, with an average of 82mg/kg body weight formononetin, 65mg/kg body weight biochanin A, 7mg/kg body weight genistein, and 3mg/kg body weight daidzein. The major compounds recovered in tissues were equol, generally in the largest concentration, and daidzein as glucuronides. The highest concentrations of equol and daidzein were found in kidney – 10-fold higher than in other tissues – and in decreasing order of concentrations in tissues liver, plasma, aorta, suprarenal glands, uterus, thyroid, and mammary gland. Lower isoflavone concentrations were detected in lung, pituitary gland, thymus, heart, muscle, olfactory lobe of brain, cerebellum, and cerebral hemisphere. The penetration into the brain was very limited. Interestingly, isoflavones were found in the thyroid. Red clover silage ingestion has been documented to stimulate thyroid hormone

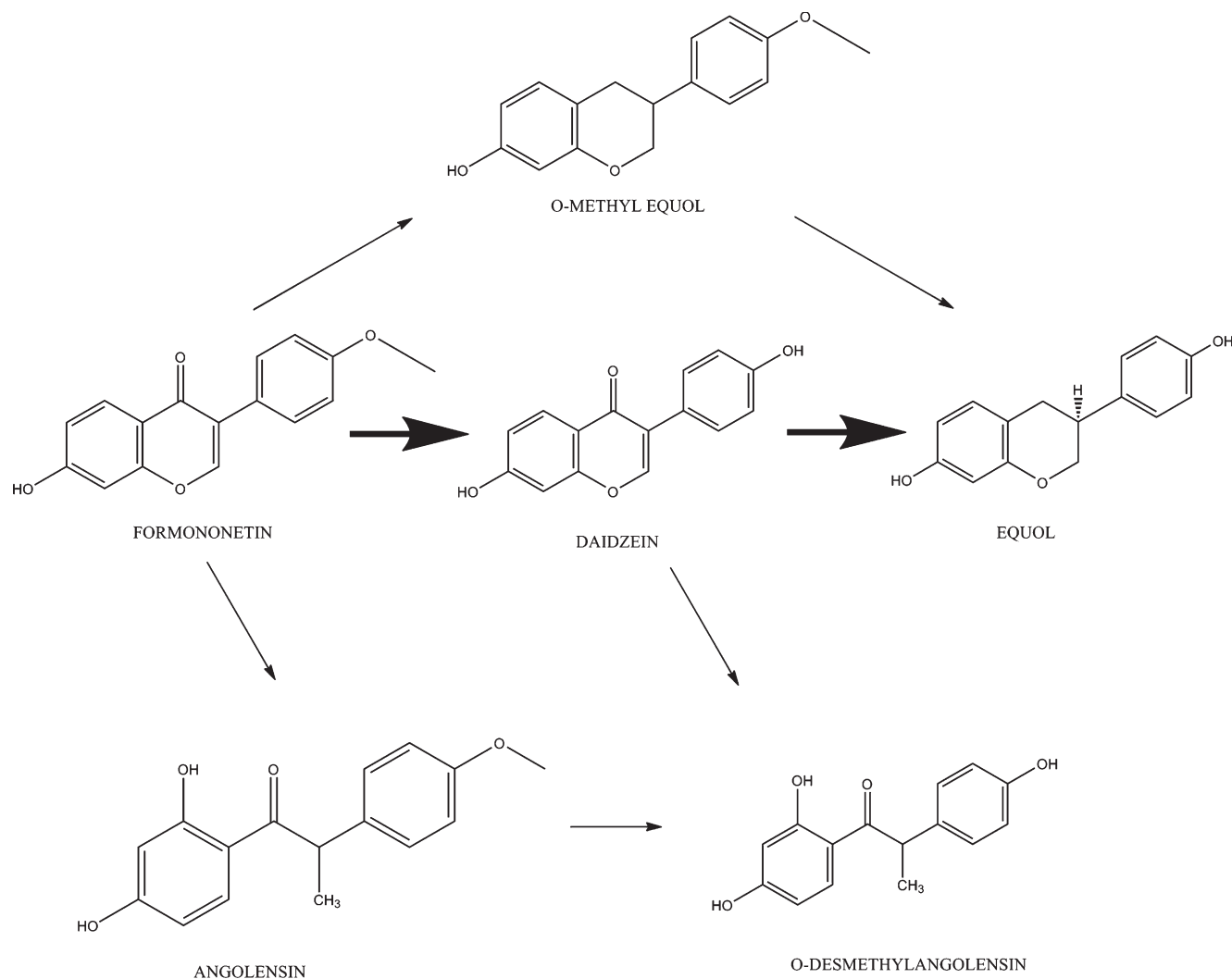


FIGURE 76.3 Metabolic pathway of formononetin via daidzein to equol in the rumen (Cox and Davies, 1988).

secretion (total and free triiodothyronine) and increase thyroid follicle size and the ER $\alpha$  immune reactivity of thyroid glands in ovariectomized ewes (Madej *et al.*, 2002). The two major isoflavones found in red clover, formononetin and biochanin A, were not recovered in tissues, which is consistent with extensive rumen metabolism of methylated isoflavones.

### Excretion

Conjugated and free metabolites are excreted in urine. A variable amount of phytoestrogens are excreted into bile and feces; for example, a greater fraction of genistein compared with daidzein is eliminated in bile and feces in rats (reviewed by Manach *et al.*, 2005). Phytoestrogens are also excreted into milk, with animal diet playing a major role in detectable concentrations.

Data indicate that animal feeds, such as soy meals, clovers, and grass/alfalfa feedstuffs, may influence milk phytoestrogen content. In a dairy cow ration of mixed red clover-grass silage, formononetin (0.3–0.5%) and biochanin A (0.2%) were the predominant isoflavones. Dairy cows fed a mixed red clover silage produced milk with high concentrations of equol (272 and 364  $\mu\text{g/L}$  or parts per billion (ppb)) and enterolactone (21 and 27  $\mu\text{g/L}$ ), metabolites of formononetin and the plant lignans (secoisolariciresinol and matairesinol), respectively (Steinshamn *et al.*, 2008). Higher equol concentrations were determined in milk from cows fed red clover silage compared with those fed white clover silage. Skimmed milk originating from organically managed Finnish dairy operations contained higher concentrations of isoflavonoids, with equol concentrations of  $411 \pm 65 \mu\text{g/L}$ , compared to that from conventionally managed dairy operations (Hoikkala *et al.*, 2007). The presence of equol

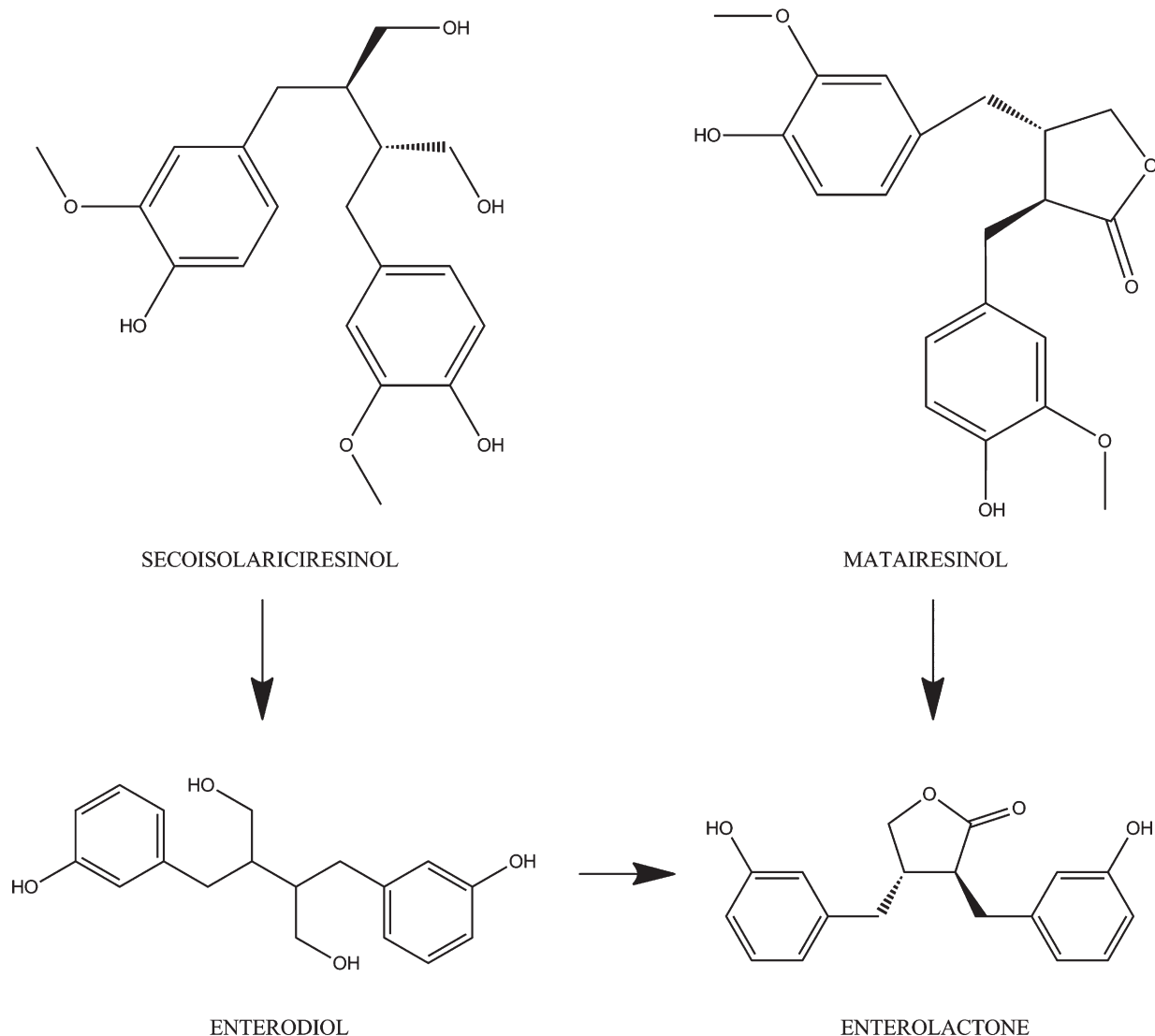


FIGURE 76.4 Schematic diagram of metabolism of plant lignans.

and enterolactone at elevated concentrations in dairy milk, from 14.1 to 293  $\mu\text{g/L}$  and 14.3 to 94  $\mu\text{g/L}$ , respectively, could be considered a health concern in children (Antignac *et al.*, 2004). Trace concentrations of methoxylated formononetin and biochanin A and hydroxylated daidzein and genistein (0.1–5.0  $\mu\text{g/L}$ ) were detected in bovine milk. Antignac *et al.* found that phytoestrogen concentrations in skimmed and full cream milk were similar, indicating that phytoestrogens are not very lipophilic compounds. For comparison purposes, the total isoflavone content of soy milk has been reported to be 6–10 mg aglucone equivalents/100 g wet weight (Chan *et al.*, 2009).

## MECHANISM OF ACTION

Reproduction is under hormonal regulation. Abnormalities in the dynamics of hormone production, metabolism, target molecule binding, and elimination can lead to alterations in the structure and/or function of the reproductive system. Estrogens influence cell growth and differentiation of both female and male reproductive tissue. They regulate the ovaries and testes, uterus, vagina, mammary glands, epididymis, and prostate gland. Phytoestrogens have been reported to affect physiological responses related to reproduction through

**TABLE 76.3** Several proposed mechanisms of phytoestrogen actions on reproduction and development

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Genomic effects through binding to estrogen receptors $\alpha$ and $\beta$ causing endocrine disruption
Non-genomic effects through binding to steroid membrane receptors
Affects metabolism through inhibition of enzymes in steroidogenesis (3 $\beta$ - and 17 $\beta$ -hydroxysteroid dehydrogenase, aromatase, 5 $\alpha$ -reductase, 17 $\beta$ -hydroxysteroid oxidoreductase type 1)
Stimulation of sex hormone-binding globulin (SHBG)
Inhibition of protein tyrosine kinase involved with signal transduction and cell proliferation
Inhibition of DNA topoisomerases I and II required for DNA replication
Inhibits matrix metalloproteinase 9 (MMP9) involved in cell growth
Down-regulates expression of vascular endothelial growth factor (VEGF) involved with growth factor genes and angiogenesis
Inhibits prostaglandin synthesis via lipoxygenase or cyclooxygenase-2 and exert antioxidant activity

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numerous mechanisms (Table 76.3). Phytoestrogens are considered weak estrogens, with an activity on the order of  $10^{-2}$  to  $10^{-3}$  of 17 $\beta$ -estradiol, but may be present in the body at concentrations 100-fold higher than those of endogenous estrogens (Adlercreutz and Mazur, 1997). A number of phytoestrogens have been shown to stimulate uterine growth in laboratory and farm animals. However, not all mouse strains were susceptible to isoflavone-induced uterine hypertrophy. The Swiss albino CD-1 mouse and ICR mouse showed no or only a slight response to phytoestrogens, respectively, which may be related to metabolism (reviewed by Kurzer and Xu, 1997). In addition, the isoflavone genistein has been demonstrated to inhibit important pathways for cellular growth and proliferation in multiple tissues.

## Estrogen receptors

Estrogens play an important role in physiological functions via a genomic mechanism. Phytoestrogens can mediate their effects by diffusing through the cell membrane and binding to specific estrogen receptors (ERs) in the target cell. After binding, the phytoestrogens do not act like typical estrogen agonists but, rather, more like selective estrogen receptor modulators (SERMs) that have differential actions as agonists or antagonists in different tissue potentially causing endocrine disruption. The differential action occurs partly from ER ligand conformational changes and the influence of co-regulator proteins (reviewed by Patisaul and Jefferson, 2010). Following phytoestrogen–receptor complex conformation changes, the complex translocates to the nucleus. Within the nucleus, the complex binds to selective regions of the DNA, the estrogen response element (ERE), and stimulates or inhibits specific genes that may result in the production of messenger RNA and subsequently new specific proteins. Numerous studies have focused on the direct effects of phytoestrogen receptor binding *in vitro*. Binding affinities to the ERs, whose subtypes are ER $\alpha$  and ER $\beta$ , vary greatly with

phytoestrogens and the various cell lines used in studies and chosen endpoints of estrogenic potency (Kuiper *et al.*, 1997, 1998). Compared to any phytoestrogen tested, the most potent activator of binding to ERs was the endogenous hormone 17 $\beta$ -estradiol. Daidzein, genistein, coumestrol, equol, and *O*-desmethylangolensin apparently bind to ERs from sheep uterine cytosol, suggesting estrogenic effects. Formononetin at physiological concentrations did not bind to the ER but could be metabolized to daidzein and eventually to equol, both of which have estrogenic properties (Kuiper *et al.*, 1998). Daidzein preferentially activated binding of ER $\beta$ , but with metabolism of daidzein to equol, both ER $\beta$  and ER $\alpha$  can be activated (Kostelac *et al.*, 2003). Phytoestrogens appear to have a predilection for greater affinity to ER $\beta$ . Studies have shown that coumestrol has a 2-fold higher affinity for ER $\beta$  than for ER $\alpha$ , and genistein has a pronounced affinity (30-fold) for ER $\beta$  (Kuiper *et al.*, 1997).

Although coumestrol may bind to both ER $\alpha$  and ER $\beta$ , it is an atypical estrogen because it does not stimulate uterine cellular hyperplasia. Coumestrol significantly increased uterine wet and dry weights in ovariectomized rats, but it did not stimulate cytosolic ER depletion or nuclear ER accumulation (Markaverich *et al.*, 1995). These findings suggest that the estrogenic effects of coumestrol may be mediated by increased sensitivity of the tissue to endogenous estradiol. An antiestrogenic effect of phytoestrogens has been proposed when high concentrations of phytoestrogens compete with endogenous estrogens and bind the ER, which blocks endogenous estrogen actions and reduces cellular growth (Rosselli *et al.*, 2000).

Differential expression of ERs has been reported in tissues, and several physiological roles have been associated with the ER subtypes (Table 76.4). Both subtypes have been found in blood vessels and in breast, uterus, and ovaries of women, but the proportions of  $\alpha$  and  $\beta$  subtypes appear to vary with estrogen target tissues, physiological and pathological status, and age of the individual. Each of these ERs may influence the function of the other, creating a complex process of



TABLE 76.4 Estrogen receptor  $\alpha$  and  $\beta$  proposed actions and distribution<sup>a</sup>

ER $\alpha$ and ER $\beta$ function in:	Normal ovarian follicular development Vascular endothelial cells Myocardial cells Smooth muscle cells Breast cells
ER $\alpha$ associated with:	Bone maturation in males and females Important role maintaining follicle-stimulating hormone and luteinizing hormone in blood More predominant in kidney, adrenal, nonpregnant human myometrium
ER $\beta$ associated with:	Bone maintenance Frontal lobe-mediated memory and learning Coumestrol and genistein bind with higher estrogenic potential Equol has modest affinity for binding More predominant in human brain, thymus, bladder, ovarian granulosa cells, testis Sertoli and germ cells, lung, bone, and pregnant term human myometrium

Based on Kuiper *et al.* (1997, 1998) and Patisaul and Jefferson (2010).  
<sup>a</sup>Distribution of estrogen receptors in tissues can change over a life span and is sexually dimorphic.

estrogenic effects in tissues in which both subtypes are co-expressed (Benassayag *et al.*, 2002). The resulting changes in physiological functions can be difficult to interpret.

Estradiol can bind with high affinity to plasma membrane forms of steroid receptors and may mediate non-genomic actions with a variety of short-term estrogen effects (Pietras and Szego, 1975; Swego, 1984). Estradiol has been shown to induce rapid changes in intracellular calcium concentrations/flux, potassium conductance, and cyclic AMP levels (reviewed by Rosselli *et al.*, 2000). The direct effects of phytoestrogens and membrane ERs have not been fully defined. Resveratrol, a stilbene phytoestrogen found in red wine, apparently binds to and increases the transcriptional activity of ER $\alpha$  and ER $\beta$ . Klinge and co-workers (2005) reported that resveratrol, at nanomolar concentrations achieved by reasonable red wine consumption, can activate membrane-initiated (non-genomic) ER signaling in endothelial cells that activate mitogen-activated protein kinases (MAPKs) involved in signaling pathways and endothelial nitric oxide synthase. Resveratrol increased nitric oxide levels in human umbilical vein endothelial cells after short-term exposure, suggesting a potential cardioprotective effect.

Impact on steroidogenesis

Certain phytoestrogens may alter key steroidal enzymes in tissues, although most studies have used *in vitro* cell

lines or purified microsomal or enzyme preparation (Lacey *et al.*, 2005). Phytoestrogens may interfere with the synthesis or metabolism of steroid hormones, such as cytochrome P450arom (aromatase), an enzyme that can catalyze the conversion of testosterone to 17 $\beta$ -estradiol and  $\Delta$ 4-androstenedial to estrone. The enzyme aromatase has a critical role in the ovary (in premenopausal women) and peripheral tissues, which are sites for estradiol synthesis in postmenopausal women and men. Using an *in vitro* assay with human breast cancer MCF-7 cells, Almstrup and co-workers (2002) reported that phytoestrogens, but not genistein, were aromatase inhibitors at low concentrations (<1 $\mu$ M) but at higher concentrations (>1 $\mu$ M) were estrogenic. The aromatase inhibition at low doses of phytoestrogens may provide antiestrogenic properties that play a role in protection against breast cancer.

Phytoestrogens may decrease endogenous estrogen concentrations through effects on the sex (or serum) hormone-binding globulin (SHBG). This binding protein has specific affinity for estrogens and androgens. Minor changes in the amount or availability of SHBG, caused by phytoestrogens, may change the free fraction of endogenous hormones in circulation either locally or systemically. The phytoestrogen enterolactone (1–10 $\mu$ M) stimulated SHBG *in vitro* with HepG2 cells (Adlercreutz *et al.*, 1992). Equol, genistein, daidzein, enterolactone, and enterodiol appear to exert a dose-dependent inhibitory effect on binding of steroids to SHBG, displacing 17 $\beta$ -estradiol or testosterone (Benassayag *et al.*, 2002). Additional mechanisms of action may affect steroid hormones. Coumestrol and, to a lesser degree, genistein have been shown to inhibit the enzyme 17 $\beta$ -hydroxysteroid oxidoreductase type 1, which converts [<sup>3</sup>H]-estrone to [<sup>3</sup>H]-estradiol in a dose-dependent manner (Mäkelä *et al.*, 1995). *In vitro* studies have reported that phytoestrogens inhibit 17 $\beta$ -hydroxysteroid dehydrogenase, converting androstenedione to testosterone, and 5 $\alpha$ -reductase, converting testosterone to the more potent dihydrotestosterone (reviewed by Whitten and Patisaul, 2001). Biochanin A displayed dose-dependent inhibition of 3 $\beta$ -hydroxysteroid dehydrogenase, an enzyme that catalyzes the conversion of pregnenolone to progesterone and androstenediol to testosterone, in primary cultures of human granulosa luteal cells (Lacey *et al.*, 2005).

Additional impact on cell functions

Angiogenesis is essential for ovarian follicle development and for tumor growth, invasion, and metastasis. Several studies have reported that phytoestrogens inhibited vascular endothelial growth factor (VEGF)-induced endothelial cell functions and signaling pathways. The

flavonoid quercetin at 5 and 50 µg/mL (concentrations higher than physiological range) inhibited VEGF production by porcine granulosa cells *in vitro* (Santini *et al.*, 2009). Santini *et al.* determined that quercetin inhibited steroidogenesis, specifically progesterone production, but not granulosa cell growth.

Genistein has been reported to inhibit ethoxyresorufin-O-deethylase (EROD) activity, part of the enzyme cytochrome P450 family (CYP1A) that is critical in the metabolism of 17β-estradiol to hydroxylated estrogen, in mammalian cell culture lines (Shon *et al.*, 2006). In addition, the activity of ornithine decarboxylase, a critical enzyme in polyamine biosynthesis and normal cell growth and proliferation, was markedly reduced after genistein treatment of MCF-7 breast cancer cells, suggesting that genistein might be of therapeutic value in preventing human breast cancer.

### Altered signal transduction and cell proliferation

Genistein may alter cell growth at several signal transduction pathways, inhibiting protein tyrosine kinase activity and down-regulating epidermal growth factor receptor autophosphorylation, which phosphorylates tyrosyl residues of membrane-bound receptors (Akiyama *et al.*, 1987), and MAPK activity and mitogen proliferation in human aortic smooth muscle cells (Dubey *et al.*, 1999). Inhibition of protein tyrosine kinases and MAPK by isoflavones may play a role in improving heart function. Genistein can inhibit DNA replication enzymes associated with cancer growth, including DNA topoisomerases I and II (required for DNA replication) and matrix metalloproteinase 9 (a matrix enzyme that can degrade a number of structural components) (Kurzer and Xu, 1997). Although inhibition of these pathways may lead to inhibited cell growth and a protective effect, a number of phytoestrogens, including coumestrol, genistein, biochanin A, daidzein, and enterolactone, can stimulate cellular proliferation of the estrogen-dependent MCF-7 human breast cancer cells at concentrations less than 1–10 µM (reviewed by Kurzer and Xu, 1997). The influence of genistein on cells *in vitro* appears to be biphasic in nature, inducing cell growth of MCF-7 cells at low concentrations and inhibiting cell growth at higher concentrations (reviewed by Rosselli *et al.*, 2000). Genistein, which shows a high affinity to ER, has been shown to inhibit growth in both ER-positive and ER-negative cell lines *in vitro*.

The antioxidant activity of phytoestrogens has been reported both *in vitro* and *in vivo*. Isoflavones inhibit lipoxygenase action and prevent sheep erythrocyte hemolysis *in vitro* and inhibit production of hydrogen peroxide in HL60 cells (reviewed by Benassayag *et al.*, 2002). Inhibition of lipoxygenase and possibly

cyclooxygenase may modulate production of prostaglandins and leukotrienes involved in inflammation, carcinogenesis, and reproduction.

## ADVERSE HEALTH EFFECTS

### Developmental effects

The effects of pre- and neonatal exposure to phytoestrogens on development in laboratory animals have been studied, with variable effects observed. Pre- and neonatal treatment of rodents with phytoestrogens has resulted in altered prepubertal or adult morphology and possible function of the uterus, vagina, ovary, breast, pituitary, and hypothalamus (reviewed by Whitten and Patisaul, 2001). *In utero* exposure to several estrogens, including genistein, down-regulated the expression of several testicular genes in the rat and mouse (reviewed by Phillips and Tanphaichitr, 2008). Both male and female rat offspring from dams treated with high levels of genistein (5000 µg) had shorter anogenital distances at birth, and females in this treatment group had a later onset of vaginal opening or puberty (Levy *et al.*, 1995). High levels of dietary coumestrol (100 mg/kg) fed to weanling female rats on days 21–24 or 22–60 caused earlier vaginal opening and irregular vaginal cycles (Whitten and Naftolin, 1992). Coumestrol treatment of neonatal female rats, given a 100 µM dose, resulted in premature uterine gland development and increased uterine weights on postnatal days 1–5, and at later ages the uterine weights and ER levels were reduced; however, if coumestrol was administered on postnatal days 10–14, the uterine gland growth was inhibited (Benassayag *et al.*, 2002).

Female neonatal rats given a subcutaneous injection of 10 µg of genistein showed an increased pituitary response to gonadotropin-releasing hormone, with higher genistein doses causing a decreased luteinizing hormone secretion on postnatal days 1–10 (Faber and Hughes, 1993). The effects of the higher doses were similar to the typical effects of estrogens in masculinizing the brain and decreasing pituitary response. Oral exposure of female CD-1 mice to genistin, the glycosylated form of genistein found in soy-based infant formulas, treated on postnatal days 1–5 caused estrogenic responses, including altered ovarian differentiation (multi-oocyte follicles), delayed vaginal opening, and, subsequently in the adult mouse, abnormal estrous cycles, decreased fertility, and delayed parturition (Jefferson *et al.*, 2009a). Jefferson *et al.* noted that the glucoside forms of isoflavones were quickly hydrolyzed to produce the aglycone forms and subsequently absorbed. The glycosylated form can be passively transported across the intestinal membrane and enter circulation by the sodium-dependent glucose transporter,

unlike passive diffusion by the aglycone form. Major contributions to infertility in genistein-treated neonatal mice were determined to be (1) delay in fertilization by an undetermined mechanism that could lead to altered developmental timing (lack of development between the two- and four-cell stage), (2) adverse oviductal environment because more than half of the embryos were lost in early embryo development, and (3) the reproductive tract (uterus not responsive to hormonal cues) was not capable of sustaining pregnancy (Jefferson *et al.*, 2009b).

Estrogen receptors are located in numerous areas of the brain, and phytoestrogens can have extensive effects. The paraventricular nucleus of the hypothalamus (PVN) is a region coordinating reproductive, social, and stress behaviors that primarily expresses ER $\beta$  (reviewed by Patisaul and Jefferson, 2010). ER $\beta$  is expressed at higher levels than ER $\alpha$  in the basal forebrain, hippocampus, and cerebral cortex (areas important for memory) in the adult. Notably, ER $\alpha$  is primarily expressed in the ventromedial nucleus (VMN) of the brain, which along with the PVN nucleus is important for initiation and regulation of sexual behavior. Patisaul and Jefferson noted that the PVN is the main site for oxytocin production, involved with social behavior and facilitation of sexual behavior. Estrogen binding to ER $\beta$  may stimulate oxytocin production from the PVN, which subsequently binds to the oxytocin receptor in the VMN, a nucleus involved in mediating the lordosis response in females. Up-regulation of oxytocin receptors involves binding to ER $\alpha$ .

The central nervous system–gonadal axis and male sexual behavior of the rat appear to be sensitive to phytoestrogens in the rat (Santti *et al.*, 1998). Altering the isoflavone dietary concentrations significantly affected both the sexually dimorphic nucleus of the preoptic area and the anteroventral periventricular nucleus in the brain of rodents (Lephart *et al.*, 2005).

Phytoestrogens may be incorporated as an alternative protein source for aquatic nutrition and have been found in discharged kraft mill effluent and sewage treatment plant effluent, with low concentrations of genistein detected that could impact fish populations. Phytoestrogens may alter sex differentiation in early development, causing a paradoxical sex reversal with increased male phenotypic sex resulting from the administration of an estrogen mimic in the diet. Increased concentrations of genistein, from 0 to 8 mg/g in the diet, fed chronically to sexually undifferentiated channel catfish (*Ictalurus punctatus*) altered gonadal sex differentiation, with increasing proportions of intersex fish and phenotypically male individuals (Green and Kelly, 2009).

Preliminary evidence indicates that soy infant formulas may exert estrogenic activity in the developing human reproductive tract. Phytoestrogens can cross the placenta and are capable of crossing the blood–brain barrier to a limited extent. The human

myometrium primarily expresses ER $\beta$  in late pregnancy and may be a target for genistein, which preferentially binds to this receptor subtype. A pilot study of female infants fed soy formula, cow milk formula, and breast milk revealed that soy milk-fed infants had re-estrogenization of vaginal cells at 6 months of age (Bernbaum *et al.*, 2008). However, an expert panel report from the National Toxicology Program and the National Institute of Environmental Health Sciences concluded in 2006 that there was insufficient human or experimental animal data to permit determination of the toxicity of soy infant formula on development or reproduction (Rozman *et al.*, 2006).

## Hormones and estrous cycles

Following adult exposures, phytoestrogens may suppress the adult hypothalamic–pituitary–gonadal axis. Data from studies of ovariectomized rodents and of humans suggest that ingestion of isoflavone-rich soy food may suppress circulating estrogen and progesterone concentrations and can attenuate the preovulatory surge of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (reviewed by Patisaul and Jefferson, 2010). Several studies have reported on the effects of soy isoflavones in premenopausal women. In a limited study during one menstrual cycle in six premenopausal women given 45 mg of isoflavones daily, the follicular phase length was increased and delayed menstruation (Cassidy *et al.*, 1994). Follicular estradiol concentrations were increased and mid-cycle surges of LH and FSH were significantly suppressed. One case report on three women described adverse effects of abnormal uterine bleeding, leiomyomas, and endometriosis related to high intakes of soy products; all of the women improved after withdrawal of soy from their diet (Chandrareddy *et al.*, 2008). A prospective cohort study of almost 20,000 non-Hispanic white women between 35 and 59 years of age, who were enrolled in the National Institute of Environmental Health Sciences Sister Study, evaluated associations of *in utero* and early life exposures and self-reporting of physician-diagnosed fibroids (D'Aloisio *et al.*, 2010). Fibroids are benign smooth muscle tumors (uterine leiomyomata) associated with reproductive problems in women. Increased risk for fibroids in early adulthood was associated with being fed soy formula during infancy, having a mother with prepregnancy diabetes, being born at least 1 month early, and growing up in lower socioeconomic conditions. The authors suggested that exposure of infants to soy formula within the first 2 months of life is a sensitive time period and can result in infant isoflavone levels more than five times higher than typical levels found in adults consuming soy-based foods.



## Infertility

Phytoestrogens have the capability to affect reproduction at many levels, from the hypothalamic–pituitary level to local levels of the ovary and uterus and testis and prostate gland (see Chapter 19, Figure 19.4). In laboratory animals, intravenous infusion of coumestrol, but not genistein, affected control of LH secretion at both pituitary and hypothalamic levels (McGarvey *et al.*, 2001). At the pituitary level, coumestrol inhibited gonadotropin-releasing hormone–LH release *in vivo*, and at the hypothalamic level, coumestrol reduced the frequency of the gonadotropin-releasing hormone pulse generator. The inhibitory effects of coumestrol on LH at the pituitary level occurred via an ER-mediated process. Additional evidence that phytoestrogens immediately affect pituitary responsiveness was found in ewes when genistein was administered directly into the central nervous system (Romanowicz *et al.*, 2004). Lower plasma LH concentrations were detected in ovariectomized ewes infused intracerebroventricularly for 6 h with two different levels of genistein at 1 and 10 µg/100 µL/h. In addition, the plasma prolactin concentrations were significantly higher in treated ewes compared with control ewes. Estrogen has been shown to be a potent stimulator of prolactin release in the pituitary lactotrophs.

Initial cases of temporary and permanent infertility in animals related to phytoestrogens occurred in sheep ingesting subterranean clover in Australia (Adams, 1995). Red clover, a livestock forage supplement, caused adverse effects in reproductive organs of ovariectomized sheep, with increased teat length (a relatively sensitive parameter), mammary gland development, and milky fluid secretions (galactorrhea) from the teats. Nwannenna and co-workers (1995) described clinical effects of edema and mucous discharge from the vulva, fluid accumulation in the uterus, elongated teats, and the presence of milky fluid in the mammary glands in ovariectomized heifers fed 20 kg of 100% red clover silage (daily intake of 35 g phytoestrogens, primarily formononetin and biochanin A) per day for 14 days. The magnitude of the pituitary response to gonadotropin-releasing hormone injections was diminished with lower LH release. The authors noted that the abnormal mounting behavior in one of the heifers was similar to the sexual behavior of cattle treated with estradiol.

Cattle seem to be less sensitive than sheep to clover forage, with temporary estrogenic signs and infertility occurring following ingestion of clover or alfalfa forages, although coumestrol is thought to have a cumulative effect. Concentrations of coumestrol of approximately 25 mg/kg (dry matter) in forage may have adverse effects on reproduction in livestock and reduce fertility (Saloniemi *et al.*, 1995), whereas dietary coumestrol concentrations of 50 mg/kg provided for more than

180 h induced uterine enlargement in rats (Whitten *et al.*, 1992). There is marked variability in the effects of phytoestrogens for different species. High concentrations of daidzein and genistein from soybeans in captive cheetah diets, with an approximate consumption of 50 mg isoflavones/day, may have been a major contributor to the decreased fertility and the veno-occlusive liver disease in the cheetah population (Setchell *et al.*, 1987).

Physiology, particularly the stage of pregnancy or cycling, appears to influence the concentrations of isoflavones (daidzein and genistein) in the plasma of heifers fed 2.5 kg soybeans (Woclawek-Potocka *et al.*, 2008). Pregnancy influenced the kinetics of the isoflavones; plasma concentrations of daidzein and genistein were significantly higher in cycling heifers than in early or late pregnant heifers. In addition, heifers that were 2 months pregnant had higher concentrations of the active metabolite equol compared to heifers that were 8 months pregnant or heifers at the mid-luteal phase of the estrous cycle. Piotrowska and co-workers (2006) found elevated concentrations of equol and *para*-ethylphenol in corpus luteal tissue and plasma of cows fed a soy diet (2.5 kg soybean/animal/day) compared with a standard diet. Metabolites of isoflavone phytoestrogens, such as equol, appear to disturb bovine corpus luteum function *in vitro* by inhibiting LH (needed for a preovulatory surge) and prostaglandin-stimulated progesterone secretion.

Uterine endometrial release of prostaglandin F<sub>2α</sub> is under regulation by oxytocin, progesterone (P<sub>4</sub>) and estradiol (E<sub>2</sub>) in ruminants and causes luteolysis and regression of the corpus luteum. In cattle and sheep, an increase in oxytocin receptors on endometrial epithelial cells is a primary initiator of luteolysis (Goff, 2004). Oxytocin has a functional role in the regulation of ovarian function in ruminants. Mlynarczuk and co-workers (2011a,b) determined that coumestrol, daidzein, and genistein stimulated the expression of several genes that are responsible for synthesis of the oxytocin precursor, neurophysin-I/OT, and post-translation synthesis of oxytocin, peptidyl-glycine- $\alpha$ -amidating monooxygenase or PGA, in granulosa and luteal cells in cattle. These phytoestrogens stimulated the secretion of oxytocin stored in bovine ovarian follicles and corpora lutea, which in cattle may result in premature luteolysis, the formation of persistent corpus lutea, and impair pregnancy maintenance in the cow.

Higher concentrations of active estrogenic metabolites (i.e., equol) in early pregnancy could lead to increased insemination rates (number of breedings) and decreased number of successful pregnancies in cattle fed soybeans at 2.5 kg/head/day (Woclawek-Potocka *et al.*, 2005a,b). The concentrations of a metabolite of prostaglandin PGF<sub>2α</sub> or PGFM were significantly higher in the soybean-fed cattle plasma through the first 21 days after ovulation and artificial insemination; the high



concentrations of PGFM were correlated with isoflavone metabolites in the plasma. Woclawek-Potocka *et al.* concluded that the soy-derived phytoestrogens and their metabolites disrupt reproduction and uterine function by modulating the ratio of  $\text{PGF}_{2\alpha}$  to  $\text{PGE}_2$ , leading to elevated, nonphysiological production of luteolytic  $\text{PGF}_{2\alpha}$  by the bovine endometrium during the estrous cycle and early pregnancy in cattle. In ruminants,  $\text{PF}_{2\alpha}$  is the major luteolytic agent, whereas  $\text{PGE}_2$  is considered luteoprotective with anti-luteolytic properties; the ratio of  $\text{PGF}_{2\alpha}$  to  $\text{PGE}_2$  is important for the development and maintenance of the corpus luteum and establishment of pregnancy.

### Male infertility

There are a limited number of reports of adverse effect of phytoestrogens on male reproductive function. In a review on soy and male reproductive function, Cederroth *et al.* (2009) stated that overall there are some indications that phytoestrogens may alter reproductive hormones, spermatogenesis, sperm capacitation, and fertility; however, there is lack of consistency in human and animal studies examining these effects. Adult male mice fed a soy-rich diet from conception to adulthood exhibited normal male behavior and were fertile but had a 25% reduction in epididymal sperm counts and a 21% reduction in litter size (Cederroth *et al.*, 2010). Irrigated red clover, containing higher estrogenic activity than non-irrigated clover, was fed with non-irrigated red clover in diets to Japanese quail chicks for 2 weeks (Rochester *et al.*, 2009). Both irrigated and non-irrigated red clover reduced chick growth compared with that of control chicks, but irrigated red clover reduced both absolute and relative testes and ovary weights and increased the relative oviduct weights, suggesting isoflavones affect avian reproductive development. Bilgoraj ganders were fed diets containing high concentrations of phytoestrogens ( $\sim 140 \mu\text{g/g}$  of diet) from soy and alfalfa meal during growth, photorefractoriness, and laying periods (reviewed by Dusza *et al.*, 2006). Semen samples analyzed from phytoestrogen-fed ganders had decreased volume of ejaculates and increased number of abnormal spermatozoa, but fertility of eggs and the percentage of normal hatching were not different in males and females fed the control and phytoestrogen diets during the breeding season.

Male goat kids fed a conventional diet supplement with red clover isoflavones (60% biochanin A), at approximately 3 or 4 mg/kg/day for 3 months, exhibited a rise in plasma-free and total triiodothyronine ( $\text{T}_3$ ) followed by a significant increase in plasma testosterone concentrations during puberty compared with those of control goats (Gunnarsson *et al.*, 2009). The increased concentration of  $\text{T}_3$  had a direct effect on pubertal Leydig cell steroidogenesis leading to increased testosterone. In

an *in vitro* experiment with pubertal rat Leydig cells, Maran and co-workers (2000) reported that  $\text{T}_3$  modulated LH-mediated secretion of testosterone and estradiol in a dose-dependent manner. Additional phytoestrogen studies have not found an effect on thyroid hormones.

Phytoestrogens have been evaluated with normal sperm to determine altered cell signaling through inhibition of tyrosine kinase. Protein tyrosine kinase is believed to have a major role in sperm function in humans and other animals through phosphorylation of tyrosine proteins on the spermatozoa and subsequent capacitation, followed by the zona pellucida-induced acrosomal reaction and penetration of zona pellucida-intact oocytes (Pukazhenthil *et al.*, 1998). Utilizing *in vitro* genistein exposure and cat spermatozoa, no effect was detected in sperm motility, but genistein inhibited the zona pellucida-induced acrosome reaction and reduced sperm penetration into the inner zona pellucida. Menzel and co-workers (2007) reported similar findings in cryopreserved bovine spermatozoa incubated with a range of genistein concentrations, from 0.74 to  $7.4 \mu\text{mol/L}$ . Genistein did not affect tyrosine phosphorylation in cryopreserved spermatozoa, but it inhibited the progesterone and ZP3-6 peptide-induced acrosomal exocytotic event or reaction and decreased sperm-zona pellucida binding, probably by a process independent of protein tyrosine kinase inhibition.

Data from castrated rats treated with  $5\alpha$ -dihydrotestosterone (DHT) and equol showed that equol bound and sequestered DHT from the androgen receptor, resulting in increased plasma concentrations of DHT (Lund *et al.*, 2004). Lund *et al.* reported that equol administration to intact male rats somewhat blocked the negative feedback effects of DHT on pituitary LH regulation, increasing circulating LH levels and reducing ventral prostate and epididymal weights, acting as an anti-androgen. Tan and co-workers (2006) used seven marmoset monkey twins to evaluate potential adverse effects of feeding human male infants soy milk formula. Male co-twin marmoset monkeys were fed soy milk formula from age 4 or 5 days for approximately 5 or 6 weeks, which resulted in normal body weights, penis length, and fertility. However, the soy-fed monkeys had significantly increased testis weights and Sertoli and Leydig cell numbers per testis. Additional studies are needed on the influence of phytoestrogens on male development and subsequent adult male reproductive and endocrine functions.

## RISK ASSESSMENT

Phytoestrogens may result in marked adverse effects on reproduction in livestock. This risk has been recognized for years in livestock, particularly in sheep and cattle

production. With regard to sheep grazing subterranean clover in Australia, temporary and permanent fertility are recognized reproductive conditions. The occurrence of adverse reproductive effects from clover or alfalfa forage consumption in livestock cannot be reliably predicted, nor can most of the factors affecting enhanced phytoestrogen concentration be controlled (e.g., weather and insect or fungal invasion). Elevated phytoestrogen concentrations in soybeans and forages can be analyzed in rations. Producers can mitigate most of the adverse effects by diluting or eliminating the forage from the ration.

In subchronic and chronic studies of oral genistein dosing in beagle dogs, the no-observed-adverse-effect level (NOAEL) was considered to be greater than 500mg/kg/day for the 4-week and 52-week studies (McClain *et al.*, 2005). The primary effects reported were in reproductive organs and included (1) increased uterine weights in female dogs in the 4-week study, (2) atrophy of the testes and prostate gland and absent spermatozoa in the epididymis in males in the 52-week study, and (3) small decreases in ovarian weights in female dogs in the 52-week study. The no-observed-effect level (NOEL) was considered to be 150mg/kg/day for the 4-week study and 50mg/kg/day for the 52-week study. A 4-week recovery period, after the 52-week study at 500mg/kg/day of genistein, resulted in no observed changes in the dogs. To assess teratogenic and fetal toxic potential of genistein in rats, McClain and co-workers (2007) conducted several *in vivo* embryo–fetal developmental safety studies using genistein by gavage, with dosages of 0–1000mg/kg/day from days 6 to 20 of gestation, and dietary admix, with dosages of 0–500mg/kg/day from days 5 to 21 of gestation, and an *in vitro* rat whole embryo culture assay (preliminary screen) using 1–100µg/mL genistein. *In vitro* genistein exposure in the embryo culture at 10µg/mL or greater resulted in anomalies that were not predictive of *in vivo* findings. A slight maternal toxicity was reported at 1000mg genistein/kg/day by gavage doses and included decreased maternal body weights and food consumption, with adverse effects in pups reported as increased pup mortality and reduced pup body weights and milk uptake. No external malformations were noted in pups, with minor visceral and skeletal variations observed at the high dose. At the high dietary admix dose of 500mg/kg/day, maternal body weight and feed consumption were reduced, and the incidence of fetal resorptions increased with a corresponding decrease in the number of live fetuses per dam. Fetal body weights were reduced, but no treatment-related teratogenic effects were detected during external, visceral, and skeletal examinations of fetuses or in body weight normalized anogenital distance. The authors concluded that on the basis of the definitive prenatal developmental safety study (oral dietary admix exposure), the NOAEL

for maternal toxicity and adverse effects on embryonic development was 100mg genistein/kg/day when given orally by dietary admix.

## TREATMENT

The focus of phytoestrogens is on both beneficial and adverse effects. Regarding the adverse impact of phytoestrogens on livestock fertility, the current recommendation is to either delete or dilute the estrogenic component of the diet. Typically, problems with fertility (irregular cycling) or mammary gland hypertrophy in cattle or horses are related to using specific cuttings of alfalfa or clover forages. Forages and soybeans can be analyzed for isoflavones and coumestrol concentrations, and nondetectable or low phytoestrogen feeds can be substituted into rations. A washout period of several weeks (4 to 6 or more weeks) will usually result in a return to normal reproductive cycling. The current phytoestrogen data for humans do not substantiate any treatment recommendations for humans.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Because of the variability in multiple parameters of exposure (dose, timing, and duration of exposure) of numerous phytoestrogens to experimental animals and humans, there are not adequate data to determine the developmental and reproductive toxicity in animals. Laboratory and livestock research should focus on the dose–response relationship of phytoestrogens (particularly isoflavones and coumestrol) on ovarian follicular development, ovarian follicle counts, and ovarian failure. The dose–response relationship for isoflavones and coumestrol in forages and potential for adverse effects of ovarian dysfunction and early embryonic death in livestock, particularly in dairy, need to be determined. Further research on the mechanisms that underlie the impact – detrimental or beneficial – of phytoestrogens on reproductive processes in humans and farm animals is necessary.

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## Poisonous plants of the United States

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### INTRODUCTION

Poisonous plants and the secondary compounds they produce cause major economic losses to the livestock industries throughout the world. Based on 1989 data, it was determined that poisonous plants cause losses of more than \$340 million annually to the livestock industry in the 17 western states of the United States (Nielsen *et al.*, 1988; Nielsen and James, 1992). This cost only considered death losses and specific reproductive losses in cattle and sheep, and only the 17 western states were included. Less obvious costs such as lost grazing opportunities, additional feed costs, increased health care costs, management changes, increased culling costs, lost weight gains, delayed or failed reproduction, and the emotional stress accompanying many poisonous plant cases were not included in the Nielsen and James analysis. When one considers these other costs, inflation, and current animal values, and when all pastures and ranges in the United States are factored in, the economic cost of poisonous plants to the livestock industry is very high. In addition, an often ignored cost is the lost biodiversity frequently resulting from invasive species, many of which are poisonous. These invasive and poisonous species are often aggressive invaders and reduce optimum utilization of private, federal, and state managed forest, range, and pasture lands. This aspect alone has far-reaching implications, not only for livestock producers but also for many other segments of society.

A frequently asked question is why do animals consume plants that may harm them or their offspring? In some cases, it is a matter of survival. For example, in the arid livestock-producing regions of the world, such

as the western United States, regions of South Africa, Australia, China, and others, browsing or grazing animals may have limited access to high-quality forage at certain times of the year and are forced to survive by grazing some poisonous species. In other instances, hay or forages harvested in areas where poisonous plants are abundant may be contaminated with a high percentage of poisonous plants, and when animals are fed contaminated hay, they may be poisoned. Poisonous plant problems are often exacerbated during periods of below normal rainfall when the abundance of grasses is reduced. Frequently, the animal's diet shifts during the season as grasses and palatable forbs mature and senesce; for example, the consumption of some poisonous plants such as lupines, locoweeds, or larkspurs, which stay green longer into the season, may increase as the season progresses. In other instances, poisoning occurs early in the season before grasses begin to grow but poisonous plants have emerged and are usually very toxic. Poisoning by plants only occurs when animals eat too much too fast or graze it over a prolonged period of time. Therefore, management strategies to control these factors can minimize losses from poisonous plants.

At the Poisonous Plant Research Laboratory in Logan, Utah, poisonous plant problems are dealt with using a multidisciplinary approach. Veterinary medicine, chemistry, toxicology, pathology, animal and plant physiology, and range management are all key in the approach to reduce losses to the livestock industry. The plants discussed in this chapter are some of the most important poisonous plants in the United States, and considerable research has been conducted to reduce their incidence of poisoning.

This chapter is not intended to be all-inclusive but focuses on some of the most economically important and geographically widespread poisonous plants to livestock producers in the United States. There are excellent comprehensive texts available with a more general and broad coverage of specific poisonous plants (Burrows and Tyrl, 2001; Knight and Walter, 2001; Keeler *et al.*, 1978 (ISOPP1); Seawright *et al.*, 1985 (ISOPP2); James *et al.*, 1992 (ISOPP3); Colegate and Dorling, 1994 (ISOPP4); Garland and Barr, 1998 (ISOPP5); Acamovic *et al.*, 2004 (ISOPP6); Panter *et al.*, 2007 (ISOPP7); Riet-Correa *et al.*, 2011 (ISOPP8)). The ISOPP series, 1–8, 1978–2011 (Proceedings of the International Symposium on Poisonous Plants), provides a worldwide view of poisonous plant research. In 2013, the ninth international symposium (ISOPP 9) will be held in Ho Hot, Inner Mongolia, China. In addition, current research information on poisonous plants from throughout the world is available in the online peer-reviewed journal, the *International Journal of Poisonous Plant Research*, by the Poisonous Plant Research Laboratory, U.S. Department of Agriculture – Agricultural Research Service. International research, both basic and applied, as well as case reports of plant poisonings and short communications are published in a spring and a fall issue each year.

## ASTRAGALUS AND OXYTROPIS SPECIES (LOCOWEEDS, NITRO SPP., AND SELENIUM SPP.)

### Locoweeds

Of all the poisonous plants in the United States, the *Astragalus* and *Oxytropis* genera cause the most losses to the livestock industry in the western states (Graham *et al.*, 2009; Cook *et al.*, 2009c). The locoweeds are those species of the *Astragalus* and *Oxytropis* genera that contain the “loco” toxin (swainsonine) and induce the classic neurological and pathological signs of “locoism.” Swainsonine is also present in other *Astragalus* species not usually considered locoweeds, such as some selenium and nitro-containing *Astragalus*. There are three toxic syndromes associated with these species: (1) locoism, caused by the indolizidine alkaloid swainsonine (1) (24 species); (2) nitrotoxins (356 taxa); and (3) species that accumulate selenium at high levels (22 species).

### Description

These species are members of the Fabaceae (Leguminosae) or pea family. This family is distinguished by its papilionaceous flower (butterfly-like) having a single large banner petal, two side petals or wings,



**FIGURE 77.1** Comparison of *Astragalus* and *Oxytropis*. *Astragalus mollissimus* (purple loco) is on the left, and *Oxytropis lambertii* is on the right.

and two lower petals fused together to form a keel. Flowers are leguminous (pea-like), few or many, in axillary racemes, and they may be blue, purple, yellow, or white in color; fruit is a legume pod of various shapes, sizes, and surfaces among the species containing one or more kidney-shaped seeds. The distinguishing feature in *Oxytropis* is the porrect beak on the keel petal, whereas the *Astragalus* have a blunt keel petal (Figure 77.1). Some *Astragalus* species have extensions of the keel forming points, but they are not turned upward as in *Oxytropis*. In addition, *Oxytropis* species are acaulescent (without a stem, all leaves basal) with leafless flowering stalks originating from the crown, whereas leaflets of *Astragalus* species grow from the multibranched stems with the flowering head on top (Figure 77.1). Leaflets are opposite and pinnately compound. Pod shape, size, and chambers are very diverse and are a principal means of distinguishing between species (Figure 77.1).

*Astragalus* is a very large and complex genus, with 354 species and 198 varieties of *Astragalus* (552 taxa) in the United States and Canada (Welsh *et al.*, 2007). *Oxytropis* is much smaller, with 22 species and 35 varieties (57 taxa). Barneby (1964) earlier described and classified 368 species and 184 varieties of *Astragalus* and 35 species of *Oxytropis* in North America. Regardless, the *Astragalus* genus is the largest of the Leguminosae family. Species and variety identification are difficult even for trained botanists. Because of obvious similarities between *Oxytropis* and *Astragalus*, some botanists treat them together as *Astragalus*. Certainly, from a toxicology perspective, both can contain swainsonine and induce the same condition in livestock; therefore, both genera are treated the same in this discussion.

### Distribution and ecology

The *Astragalus* and *Oxytropis* are worldwide in their distribution and toxic effects on livestock. Table 77.1 lists those species in the western United States suspected

TABLE 77.1 Locoweed (*Astragalus* and *Oxytropis*) species, habitat, and distribution and *Astragalus* species containing nitro-toxins or accumulating selenium<sup>a</sup>

Species	Common name	Habitat	Distribution
<i>A. allochrous</i>	Rattleweed	Desert grassland	AZ, NM
<i>A. asymmertricus</i>	Horse loco	Annual grasslands	CA
<i>A. bisulcatus</i> <sup>b</sup>	Two-grooved milkvetch	Limestone, shale, high in Se	MT, ND, WY, CO, NM, UT
<i>A. didymocarpus</i>		Creosote deserts	CA, AZ, NV
<i>A. drummondii</i> <sup>b</sup>	Drummond milkvetch	Prairies, sage, oak, P/J	MT, WY, CO, NM, UT
<i>A. emoryanus</i> <sup>c</sup>	Red stem peavine	Creosote, mesquite, P/J	NM, TX
<i>A. humistratus</i>	Ground cover milkvetch	P/J woodlands	AZ, NM
<i>A. lentiginosus</i>	Spotted locoweed	Salt-desert shrub, sage, P/J	AZ, UT, NV, ID
<i>A. lonchocarpus</i>	Great rushy milkvetch	P/J woodlands	CO, UT, AZ, NV
<i>A. missouriensis</i>	Missouri milkvetch	Shortgrass prairies	Canada to TX
<i>A. mollissimus</i>	Woolly loco	Shortgrass prairies	CO, KA, OK, TX, NM
<i>A. nothoxys</i>	Beaked milkvetch	Oakbrush, P/J woodlands	AZ
<i>A. oxyphysus</i>	Diablo loco	Desert grasslands	CA
<i>A. praelongus</i> <sup>b</sup>	Stinking milkvetch	Sandstone, shale high in Se	UT, NM, AZ
<i>A. pubentissimus</i>	Green river milkvetch	Salt-desert shrub	CO, WY, UT
<i>A. purshii</i>	Pursh loco	Sagebrush, P/J woodlands	WY, MT, ID, NV
<i>A. pycnostachyus</i>	Brine milkvetch	Salt marshes and beaches	CA
<i>A. tephrodes</i>	Ashen milkvetch	Oakbrush, P/J woodlands	AZ, NM
<i>A. thurberi</i>	Thurber milkvetch	Creosote, oak, P/J woodlands	AZ, NM
<i>A. wootoni</i>	Garbancillo	Creosote desert	AZ, NM, TX
<i>O. besseyi</i>	Red loco	Gravelly hill tops	MT, WY
<i>O. campestris</i>	Yellow loco	Prairies, mountain meadows	MT, Canada
<i>O. lambertii</i>	Lambert locoweed	Short- and mid-grass prairies	MT, ND, SD, WY, CO, NM
<i>O. sericea</i>	White locoweed	Rocky soils, foothills, and mountains	MT, SD, WY, CO, NM, UT
<b>Nitro-toxins</b>			
<i>A. atropubescens</i>	Kelsey milkvetch	Gravelly benches	ID, MT
<i>A. Canadensis</i>	Canada milkvetch	Introduced	Throughout U.S.
<i>A. convallarius</i>	Rushy milkvetch	Sagebrush	ID, NV, UT, WY
<i>A. emoryanus</i>	Red-stem peavine	Shortgrass prairies	AZ, NM, TX
<i>A. falcatus</i>		Introduced	
<i>A. miser</i>	Timber milkvetch		
var. <i>serotinus</i>	Columbia milkvetch	Rough fescue grasslands	BC Canada
var. <i>hylophilas</i>	Yellowstone milkvetch	Sagebrush/timber	WY, MT
var. <i>oblongifolius</i>	Wasatch milkvetch	Mountains, sagebrush	UT, CO
<i>A. pterocarpus</i>	Winged milkvetch	Sagebrush	CA, NV
<i>A. tetrapterus</i>	Four-winged milkvetch	Sagebrush	CA, NV
<i>A. toanus</i> <sup>d</sup>	Toano milkvetch	Salt-desert shrub	Great Basin
<i>A. whitneyi</i>		Sierra Mountains	CA, NV
<b>Selenium accumulators</b>			
<i>A. albulus</i> <sup>d</sup>	Cibola milkvetch	Salt-desert shrub	Northwestern NM, AZ
<i>A. beathii</i> <sup>c</sup>	Beath's milkvetch	Badlands	AZ, NV, UT
<i>A. bisulcatus</i>	Two-grooved milkvetch	Sagebrush	MT, WY, ND, SD, CO, UT, NM
<i>A. crotalariae</i> <sup>d</sup>	Rattle box milkvetch	Desert	AZ, CA
<i>A. cutleri</i> <sup>d</sup>			
<i>A. debequaeus</i> <sup>d</sup>			
<i>A. eastwoodiae</i> <sup>d</sup>	Eastwood's milkvetch	Badlands	Four Corners
<i>A. flavus</i>	Yellow milkvetch	Alkaline soils	Colorado Plateau
<i>A. grayii</i> <sup>d</sup>	Gray's milkvetch	Badlands	WY, MT
<i>A. moencoppensis</i>	Moenkopi milkvetch	Badlands	UT, AZ
<i>A. nelsonianus</i> <sup>d</sup>		Alkaline flats	WY
<i>A. oocalycis</i> <sup>d</sup>		Sagebrush	CO, NM
<i>A. osterhouti</i>	Osterhout milkvetch	Clay hills	Grand Co., CO
<i>A. pattersoni</i>	Patterson milkvetch	Oakbrush	Rocky Mountains
<i>A. pectinatus</i>	Tine-leaved milkvetch	Northern prairies	MT, ND, WY, CO, KA
<i>A. praelongus</i>	Stinking milkvetch	Badlands	Four Corners
<i>A. racemosus</i>	Alkali milkvetch	Badlands	Plains states
<i>A. sabolus</i>	Cisco milkvetch	Badlands	Eastern UT
<i>A. saurinus</i> <sup>d</sup>	Dinosaur milkvetch	Badlands	Eastern UT
<i>A. sophoroides</i> <sup>d</sup>	Painted desert milkvetch		AZ
<i>A. toanus</i> <sup>d</sup>	Toano milkvetch	Salt-desert shrub	Great Basin

P/J, pinyon-juniper.

From Ralphs *et al.* (2003) and Welsh *et al.* (2007).<sup>a</sup>Many varieties, especially of *A. lentiginosus*, *A. mollissimus*, and *O. sericea*, have been referred to as separate species in the past.<sup>b</sup>Also contains selenium.<sup>c</sup>Also contains nitro-toxins.<sup>d</sup>Minor species.



of field cases of poisoning or that have been shown by chemical analysis to contain swainsonine (Molyneux *et al.*, 1991).

Species of *Astragalus* and *Oxytropis* occur in every major plant community. However, livestock poisoning is erratic due to the cyclic nature of the locoweed populations. Locoweeds have different survival strategies that allow perpetuation of the species through long-term climatic cycles and short-term weather conditions (Ralphs *et al.*, 2003). Climate controls the establishment and growth of these plants by the amount and timing of precipitation. The following are the three main survival strategies:

- 1 Annual plants avoid drought by seed dormancy through dry cycles and germinate in years when sufficient moisture is available (winter annuals such as *A. wootonii* and *A. emoryanus*).
- 2 Biennial or short-lived perennial plants exhibit opportunistic survival strategies by relying on both timely and adequate moisture for germination, growth, flowering, and seed set. Seeds germinate in fall following autumn rains, persist over winter, and flower in spring. If sufficient moisture is available, they will remain for 2 or 3 years until the next drought occurs (*A. mollissimus*, *A. lentiginosus*, and *A. pubentissimus*).
- 3 Long-lived perennial plants exhibit a stress-tolerant survival strategy. They have deep taproots that can access deep stored water. They grow where moisture is more abundant and more regularly available. The plants flower and produce seed for many years following initial establishment, although they too may die out during extended droughts (*O. sericea* and *O. lambertii*).

The seed bank in the soil supports these cycles. The seeds have hard coats and remain viable for many years, thus providing an ecological advantage to exploit environmental conditions and maintain the “boom and bust” population cycles (Ralphs *et al.*, 2003). Livestock poisonings follow these cycles, often in catastrophic proportions.

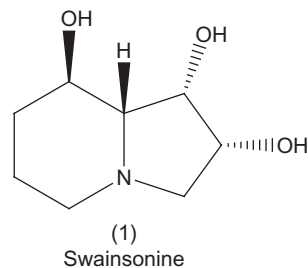
### Toxicology

Locoweed poisoning was one of the first poisonous plant problems recognized by stockmen and reported as early as 1873. Extensive stock losses in the western United States eventually led to the establishment of a field station in Hugo, Colorado, in 1905 to study the problem. Research from 1905 to 1907 determined that certain species of the *Astragalus* and *Oxytropis* genera were responsible, and this research was published in 1909 as a U.S. Department of Agriculture bulletin titled “The Locoweed Disease of the Plains” by C.D. Marsh.

There are numerous effects of locoweed on animals, but the classic syndrome from which the term “locoism” derived is one of neurological dysfunction. The disease is a chronic one developing after weeks of ingesting locoweeds and beginning with depression, dull-appearing eyes, incoordination progressing to aberrant behavior including aggression, staggering, solitary behavior, and emaciation and ending in death if continued consumption is allowed. Other problems associated with locoweed ingestion include reproductive failure, abortion, birth defects, weight loss, and enhanced susceptibility to brisket disease at high elevations (Panter *et al.*, 1999b).

Locoweed poisoning affects all animals, but because of the transient nature of the poisoning, animals removed from the locoweed early in the toxicosis will recover most of their function and may be productive animals. In the final stages of locoism, central nervous system tissue shows swelling of axonal hillocks (meganeurites) and growth of new dendrites and synapses. This altered synaptic formation in nervous tissue in severely affected animals is permanent and may be the cause of some irreversible neurological signs. Because of neurological dysfunction and apparent permanence of some lesions in the nervous system, horses are believed to be unpredictable and therefore unsafe to use for riding, but they may remain reproductively sound once they have recovered from the poisoning.

### Toxin



The toxin in locoweeds is the indolizidine alkaloid swainsonine (**1**). First isolated from the Australian plant *Swainsona canescens* (Colegate *et al.*, 1979), swainsonine and its N-oxide were isolated and identified soon thereafter from the locoweeds (Molyneux and James, 1982). Remarkably, in 1909, C.D. Marsh made the connection between the clinical and pathological syndrome of locoweed poisoning and a similar condition reported in Australian livestock called “peastruck.” Confirmation feeding trials in New South Wales with Darling pea (*Swainsona* spp.) were reported a few years before Marsh reported the details of locoweed poisoning. Ironically, the toxin (swainsonine) was isolated and characterized from *S. canescens* and reported in Australia in 1979 (Colegate *et al.*, 1979) and subsequently identified and

isolated from locoweeds (*Astragalus* and *Oxytropis*) in the United States and reported in 1982 (Molyneux and James, 1982).

The isolation and characterization of swainsonine from *S. canescens* resulted from investigations by a multi-disciplinary research team in Australia whose members were experienced in various aspects of lysosomal storage diseases in domestic animals (Dorling *et al.*, 1989). This investigation concentrated on the details of the pathogenesis of the toxicosis enabling the research team to elucidate the mechanism of action, which provided the bioassay method for toxin isolation and identification. A review of the historical aspects can be found in Dorling *et al.* (1989). In essence, the locoism syndrome is a lysosomal storage disease in which  $\alpha$ -mannosidase is inhibited, resulting in prevention of hydrolysis of mannose-rich oligosaccharides in cells and accumulation of these oligosaccharides resulting in cellular dysfunction. Swainsonine has also been identified with mixtures of other glycosidase-inhibiting polyhydroxy alkaloids in toxic species of *Ipomoea*, *Sida*, *Solanum*, *Physalis*, and *Convolvulus* (Asano *et al.*, 1995; Haraguchi *et al.*, 2003). Most organ systems are affected, but some are more susceptible, such as glandular and neurological tissues. Comparative pathology of the glycosidase inhibitors swainsonine, castanospermine, and calystegines A3, B2, and C1 in mice was reported (Stegelmeier *et al.*, 2008). Although there were similar vacuolar changes, there were differences in the organ systems involved and minor changes in protein glycosylation, and it was determined that in mice calystegines and castanospermine are less toxic than swainsonine.

It has been discovered that swainsonine in *Astragalus* and *Oxytropis* species is produced by a fungal endophyte, *Undifilum oxytropis* (formerly called *Embellesia oxytropis*). A positive correlation was shown to exist between swainsonine concentrations found in the plant and concentrations of swainsonine produced by the endophytic fungus cultured from the same plant (Pryor *et al.*, 2009; Cook *et al.*, 2009a). This same correlation was demonstrated for *Oxytropis glabra*, an important poisonous plant in Inner Mongolia steppe (Ping *et al.*, 2009). Major locoweeds were screened for the presence of endophyte and swainsonine content using both culture methods and polymerase chain reaction (PCR; Ralphs *et al.*, 2008). PCR was demonstrated to be the most sensitive method of detecting the endophyte because the endophyte in plants with very low levels of swainsonine (<0.01%) was not detected by culture methods, only PCR. Oldrup *et al.* (2010) demonstrated that *U. oxytropis* is transferred by seed and the endophyte resides in the parenchyma layers of the seeds. Seedlings of *Astragalus lentiginosus* produced in embryo culture without seed coats did not contain swainsonine or the fungus. Plants produced from whole seed contained *Undifilum* in

both foliage and root tissues. Simulated environmental stresses, including high temperature, low and high pH media, nutrient-deficient media, and polyethylene glycol-amended media, impacted both dry mass and swainsonine levels.

Research results have shown that inhibition of  $\alpha$ -mannosidase is relatively transient and quickly reversible once animals stop eating locoweed (Stegelmeier *et al.*, 1994). Blood serum clearance of swainsonine is rapid (half-life of 20h); thus, the effects of locoweed should be reversible if tissue damage has not become extensive and permanent. This suggests that intermittent grazing of locoweed should be an effective means of reducing locoweed poisoning. There is also an apparent threshold dosage where severity of cell damage is more time dependent than dosage dependent. Once the threshold dosage is reached, which appears to be relatively low (0.35mg/kg in the rat), eating more locoweed does not accelerate the toxicosis. Therefore, increasing animal numbers on loco pastures and reducing time of grazing is also a logical method to reduce adverse effects.

Many locoweeds are biennials or perennials that flourish periodically under optimum environmental conditions. Historically, losses are regional and sporadic, with large regional economic impact. Individual cases of significant losses are frequent and reported in James and Nielsen (1988). In a single case, 25% of more than 500 mother cows of a university foundation herd either aborted or apparently resorbed their fetuses after grazing pastures with *O. sericea*. Necropsy of aborted fetuses showed pathological lesions characteristic of locoweed poisoning, and 50 calves born alive showed outward signs of toxicity (James, personal communication, 1994). Follow-up communication indicated that locoed calves were immunologically compromised, and 3 or 4% of these calves died after weaning. The remainder of the loco calves remained approximately 200 pounds less than calves not exposed to locoweed even though after the first 28 days in the feedlot, the rate of gain was approximately the same.

### Conditions of grazing

The early literature suggested that locoweeds were distasteful and animals were forced to start eating them because of hunger. However, once started, animals seemed to become addicted to locoweeds. Recent research showed that locoweeds are not addicting but, rather, are relatively more palatable than associated forages during various seasons of the year.

Preference for locoweed is relative to what other forage is available. Many locoweeds are cool-season species that green up and start growth early in the spring, flower, set seed and go dormant in summer, and then resume growth in fall. Livestock generally prefer the

green, growing locoweeds to dormant grass. Sheep preferred the regrowth foliage of Green River milkvetch to dormant grasses during late fall and early winter on desert range in eastern Utah. Horses selected green spotted locoweed instead of dormant grasses in spring in Arizona (Pfister *et al.*, 2003). Cattle readily grazed Wahweap milkvetch in proportion to its availability on desert winter range in southeastern Utah. In a series of grazing studies in northeast New Mexico, cattle readily grazed white locoweed in March–May but stopped grazing it in June as warm-season grasses became abundant and white locoweed matured and became coarse and rank. Stocker cattle grazed white locoweed on short-grass prairies in May and early June, but the weight loss continued throughout the summer, even though they were not eating locoweed. On mixed-grass prairies on the eastern foothills of the Rocky Mountains in northern Colorado, cattle ceased grazing white locoweed when it matured following flowering in mid-June and became rank and unpalatable. However, they continued to graze it throughout the subsequent summer when abundant summer precipitation caused locoweed leaves to remain succulent (Ralphs *et al.*, 2001).

#### ***Prevention of poisoning and management recommendations***

Prevention of poisoning remains a matter of management strategy adapted to individual grazing programs to minimize grazing of locoweed plants (Graham *et al.*, 2009). Currently, no broad management schemes or methods of treatment are known to generally prevent locoweed poisoning. Management strategies for individual operations have been developed once the grazing practices and options are identified, allowing utilization of the particular range and yet minimizing losses. It was determined that cattle generally rejected woolly loco even under extreme grazing conditions, but once they were forced to start eating it, they continued to graze it and became intoxicated. Ranchers should watch for these “loco eaters” and remove them to clean pastures. Shortage of feed with high grazing pressure, social facilitation (loco eaters teaching non-loco eaters to eat loco), or supplementing with alfalfa hay or cubes may compel cattle to start grazing woolly locoweed. White locoweed is more palatable than woolly locoweed and is green before spring grasses begin to grow in northeastern New Mexico. Therefore, cattle readily graze white loco in early spring while grasses are dormant, and once green grass starts to grow, cattle switch off of loco. Recommendations include creating loco-free pastures through spraying, fencing, or selection of low loco-infested pastures for early spring grazing and also to provide a place to move the identified loco-eaters. This practice appears to reduce the impact of locoweed on these ranges.

Many minerals, feed additives, and clay minerals have been investigated to prevent poisoning, but none have been effective. The poisonous plant literature is filled with statements that native livestock are less likely to be poisoned than new, inexperienced livestock. Locoweed poisoning does not follow this general trend. Cattle that are familiar with locoweed will likely select it first. Early observations by C.D. Marsh as early as 1909 suggested that black cattle and black-faced sheep were more inclined to be poisoned by locoweed than were white-faced cattle and sheep. In a grazing study comparing breeds, Brangus steers consumed more locoweed than did Hereford and Charolais steers. The gregarious nature of Brangus cattle may have facilitated the social acceptance of locoweed among the steers.

Livestock should be denied access to locoweeds during critical periods when they are relatively more palatable than associated forages. On short-grass prairies of northeastern New Mexico, stocker cattle should not be turned onto locoweed-infested rangelands until warm season grasses start growth in late May or early June. Cattle on rangeland year-round should be removed from locoweed-infested areas in the spring when it is green and growing and warm-season grasses remain dormant. They can be returned to locoweed-infested pastures in summer when warm-season grasses are abundant.

Most locoweed species are endemic, growing only in certain habitats or on specific soils. Fences could be constructed on soil or vegetation boundaries to provide seasonal control of grazing. Reserving locoweed-free pastures for grazing during critical periods in spring and fall can prevent locoweed poisoning.

Locoweed-free areas can be created by strategic herbicide use. White locoweed is most susceptible to Clopyralid, but Picloram and Escort are also effective. However, natural population cycles should be considered to determine the practicality of spraying large areas and the potential lifetime of control. With the abundant seed bank in the soil, locoweeds are sure to germinate and reestablish when environmental conditions are favorable.

Animals that start eating locoweed may influence others to start. Social facilitation or peer pressure is a very strong influence inducing others to start eating locoweed (Ralphs *et al.*, 1994). Graham developed the “eat and pull” management strategy, whereby livestock should be watched closely and removed if they start eating locoweed to prevent poisoning and prevent them from influencing others to start.

Grazing pressure can also force cattle to begin grazing locoweed when they run short of desirable forage (Ralphs *et al.*, 1994). Ranchers should not overstock locoweed-infested ranges but, rather, should ensure adequate forage is always available. Improper use of

some grazing systems can cause livestock to graze locoweed. Rest-rotation grazing systems are designed to force livestock to uniformly graze all forage in a pasture. This caused cattle and horses to start grazing spotted locoweed in western Utah. Changing to a three-herd, four-pasture deferred rotation grazing system stopped locoweed poisoning by reducing the grazing pressure and allowing the cattle to select alternative forages in preference to white locoweed. The heavy grazing pressure associated with short-duration grazing systems may also induce poisoning problems.

Conditioned food aversion can be used as a management tool to train animals to avoid grazing locoweed. In the conditioning protocol, animals are brought into a pen and fed fresh-picked locoweed, and then lithium chloride (an emetic that causes gastrointestinal distress) is administered by stomach tube. The animals associate the induced illness with the taste of the plant and subsequently avoid eating it. Naive animals that are unfamiliar with the target plant form strong and lasting (>3 years) aversions following a single dose. Averted animals must be kept separate from nonaverted animals on locoweed areas to prevent social facilitation from extinguishing the aversions. Aversion conditioning may be feasible where losses are heavy and persist year after year.

### Summary

Locoweed is the most widespread poisonous plant problem in the western United States. Knowledge of sites where locoweeds grow and environmental conditions when they cause problems is necessary to manage livestock and prevent poisoning. Locoweeds are relatively palatable, and many locoweeds are the first plants to start growing in the spring and they may also regrow in the fall. Livestock generally prefer the green-growing locoweeds to other forage that is dormant in the fall, winter, and spring. The most effective management strategy is to deny livestock access to locoweeds during critical periods when they are more palatable than associated forage. Reserving locoweed-free pastures or controlling existing locoweed populations with herbicides can provide "safe" pastures for critical periods. Watching animals closely and removing those that begin eating locoweed can prevent further intoxication and prevent them from influencing others to start. Finally, conditioned food aversion is an effective practice to train animals to avoid eating locoweeds, and it may be economical where losses are large and persistent. Good range management and wise grazing strategies can provide adequate forage for livestock and avoid critical periods of the year when locoweed is relatively more palatable than associated forages.

## Nitro-containing *Astragalus* (milkvetches)

There are more than 260 species and varieties (356 taxa) of nitro-containing *Astragalus* in North America (Barneby, 1964; Welsh *et al.*, 2007). They are frequently referred to as milkvetches, as are some of the other *Astragalus* species. Nitro-toxins are therefore the most common toxin in the *Astragalus*, followed by swainsonine (loco) and selenium. Major livestock losses occur in many regions of the western United States. These plants are very diverse and concentrated on the deserts, foothills, and mountains of the west.

### Description

The description of these plants is the same as that of the locoweeds. The milkvetches emerge from late April to June depending on elevation and snow cover. After seed dispersal in July or August, the stems and leaves become dry and less dangerous as toxicity and palatability are reduced.

### Distribution

The aliphatic nitro-containing *Astragalus* are distributed throughout North America, with substantial livestock losses occurring in the 17 western states where cattle or sheep are concentrated in areas of milkvetch infestation (Table 77.1). There are examples of a number of *Astragalus* spp. such as *A. cicer* that are good forages and apparently contain low levels of the toxins discussed. Because of the extent of this group of *Astragalus*, only a few of those implicated in poisonings are mentioned here. For a more comprehensive review of the nitro-containing *Astragalus*, the reader is referred to Williams and Barneby (1977). Examples of these include *A. emoryanus* (emory milkvetch) in New Mexico, Texas, and Arizona (*A. emoryanus* also contains swainsonine); *A. tetrapteris* (four-winged milkvetch) in Oregon, Utah, Nevada, and Arizona; *A. pterocarpus* (winged milkvetch) in Nevada; and *A. miser* var. *serotinus*, *A. miser* var. *oblongifolius*, and *A. miser* var. *hylophylus* (collectively referred to as timber milkvetch) in western Canada, Montana, Idaho, Utah, Colorado, and Wyoming.

### Toxicology

The nitro-containing *Astragalus* species cause an acute and chronic type of poisoning in sheep and cattle. The acute form results in weakness, increased heart rate, respiratory distress, coma, and death. Although blood methemoglobin is high (induced from nitro-toxin metabolism to nitrites) and a contributing factor to the respiratory difficulties, administration of methylene blue in cattle does not prevent death. Therefore, the methemoglobinemia is apparently not the primary cause of death. The chronic form is the most frequent form of poisoning



observed and follows a course of general weakness, incoordination, central nervous system involvement resulting in knuckling of the fetlocks, goose stepping, clicking of the hooves, "cracker heels" progressing to paralysis, and death. A respiratory syndrome is also present in the chronic and acute forms, with emphysema-like signs causing the animals to force respiration: "roaring disease." Sheep manifest the respiratory syndrome more than the central nervous syndrome and are more resistant to poisoning compared to cattle.

The toxic principles are  $\beta$ -D-glycosides of 3-nitro-1-propanol (NPOH) or 3-nitropropionic acid (NPA). The glycoside conversion occurs more readily in the ruminant because of the microflora and is apparently the reason for increased toxicity in ruminants. The glycoside (miserotoxin) is metabolized to the highly toxic NPOH in the gastrointestinal (GI) tract of ruminants (Williams *et al.*, 1970). Thus, NPOH is absorbed in the gut and apparently converted to NPA by the liver. Further metabolism yields inorganic nitrite and an unidentified metabolite that may be involved in toxicity. It appears that NPOH is more rapidly absorbed from the gut than is NPA; therefore, forage containing the alcohol form is the most toxic.

#### Prevention and treatment

There is no preferred treatment for milkvetch poisoning, although treatment with methylene blue appears to reverse the methemoglobinemia but does not prevent death in cattle. Oxidation of NPOH to NPA was prevented if alcohol dehydrogenase was saturated with ethanol or inhibited with 4-methylpyrazole before NPOH was given. This suggests that NPOH is a good substrate for the enzyme alcohol dehydrogenase. This information could be useful in acute cases; however, its value in treatment of poisoning in the field is unknown.

Livestock losses can be reduced by decreasing the density of the *Astragalus* species with herbicides or avoiding grazing livestock on infested areas when the plant is most poisonous. Wasatch milkvetch contains the highest concentration of miserotoxin from bloom to immature pod stage of growth. Nitro compounds are found in all parts of the plant, but the leaves contain the highest concentration. Once the leaves begin to dry and lose their green color, the nitro levels drop very rapidly and the plant is relatively nontoxic. However, the toxins in plants pressed green and preserved in herbaria appear to remain stable for years (Williams and Barneby, 1977). Herbicide treatment decreases the density of plants and also decreases the toxicity of the plants once they start to dry; therefore, spraying milkvetch appears to be the best method to reduce losses and still utilize infested ranges.

### Seleniferous *Astragalus*

Approximately 22–24 species of *Astragalus* known to accumulate selenium (Se) have been identified (Rosenfeld and Beath, 1964; Welsh *et al.*, 2007). These are less numerous and more geographically restricted than the nitro-containing species. Many of these species are referred to as Se-indicator plants because they only grow on soils high in bioavailable selenium; therefore, they are helpful in locating and identifying areas or soils high in selenium. The *Astragalus* are generally deep rooted plants and may bring selenium from deeper soil profiles unavailable to other plants so that it is available and can subsequently be taken up by grasses and other forbs. It is these facultative accumulators that create most of the subacute or chronic toxicity problems for livestock.

#### Description

The selenium-containing *Astragalus* species appear similar to those of locoweed and nitro-containing species as discussed previously. One identifying feature of the selenium-containing *Astragalus* species is an unpleasant garlic-like odor of the volatile selenium compound dimethyl diselenide, especially if picked and allowed to sit in a warm car or window. The Se-indicator plants are generally considered to be unpalatable to livestock because of their high selenium content. Some *Astragalus* species have been shown to accumulate selenium at concentrations of up to 10 mg Se/g (dry weight) while growing on soils containing 2–10  $\mu$ g Se/g. The majority of selenium in accumulator plants is found as organic methylselenocysteine and selenocystathionine or as inorganic selenate. Many selenium-containing *Astragalus* may also contain other toxins; for example, *A. praelongus* and *A. bisulcatus* contain swainsonine (the loco toxin), and *A. toanus* contains nitro-toxins and swainsonine in addition to selenium.

#### Distribution

The selenium-containing *Astragalus* species are limited in their distribution to geographical locations and soil sites of high selenium (Table 77.1), of which most are alkaline in nature, causing selenium to be oxidized and making it soluble and available to plants. Major seleniferous areas of the west are in North and South Dakota, Montana, Wyoming, Colorado, and Utah, with minor isolated pockets of seleniferous soils in most of the other western states.

The *Astragalus* species most associated with selenium poisoning include *A. bisulcatus* (two-grooved milkvetch), *A. praelongus* (stinking milkvetch), *A. pattersonii* (Patterson milkvetch), *A. pectinatus* (tiny-leaved milkvetch), and *A. racemosus* (alkali milkvetch).

### Toxicity

With selenium poisonings, one may observe acute, sub-acute, or chronic selenosis depending on the daily dose and duration of exposure. Acute cases of selenium poisoning are rare and usually involve animals that have been exposed by one of three methods. First, livestock graze forages that have accumulated selenium from seleniferous soils. Second, selenium toxicosis occurs from environmental contamination from agricultural drain water, reclaimed soils from phosphate or ore mining, or from fly ash. Third, acute selenosis can be caused by accidental overdosing with organic selenium or Bo-Se in the treatment of white muscle disease or by misformulated feed mixes. The signs of acute selenium poisoning include diarrhea, unusual postures, increased temperature and heart rate, dyspnea, tachypnea, respiratory distress, prostration, and death (Tiwary *et al.*, 2006). Gross pathological findings are usually limited to pulmonary congestion and hemorrhage and pulmonary edema. Histologically, multifocal myocardial necrosis and pulmonary alveolar vasculitis are common (Tiwary *et al.*, 2006).

Chronic selenium poisoning is common and referred to as alkali disease because most areas with high concentrations of available selenium are alkaline in nature. Chronic selenosis occurs from prolonged ingestion of seleniferous forages containing 5–40 ppm Se. Clinical signs include rough coat, hair or wool loss, poor growth, emaciation, abnormal hoof growth and lameness, dermatitis, and depressed reproduction (Rosenfeld and Beath, 1964; Raisbeck, 2000). In swine, a condition of paralysis (poliomyelomalacia or polioencephalomalacia) often occurs with cervical or lumbar involvement (Panter *et al.*, 1996b). The description of a second chronic syndrome in cattle called “blind staggers” has been redefined and is now believed to be polioencephalomalacia induced by high sulfate water or high sulfate forage sources.

Selenium is found in plants in both inorganic and organic forms. The organic forms are more bioavailable than the inorganic forms, resulting in higher tissue concentrations when administered at equivalent doses (Tiwary *et al.*, 2006; Davis *et al.* 2011). Although a dramatic difference in tissue selenium uptake between organic (selenomethionine) and plant (*A. bisulcatus*) forms and inorganic (sodium selenate) forms occurs, the clinical and pathological syndromes are similar – that is, poliomyelomalacia in pigs (Panter *et al.*, 1996b) and pulmonary edema and hemorrhage in sheep (Tiwary *et al.*, 2006; Davis *et al.* 2011).

### Prevention of poisoning

There is no treatment for selenium poisoning except removal of the source, allowing spontaneous recovery

in chronic cases. Monitoring soils in a particular area and understanding the plant communities can provide the management information to avoid poisoning. In areas where selenium is a problem, many ranchers have switched to grazing steers because of decreased reproductive efficiency in cows. Sheep appear to be more resistant to chronic selenosis compared to cattle and are better adapted for some of these ranges. However, sheep are sensitive to acute selenium poisoning, as was observed when a large number of sheep died within days after grazing on mine reclamation sites that contained very high soil and plant selenium concentrations (Panter, personal communications, 2004). Monitoring for selenium concentrations and forms in soil, vegetation, as well as animal tissues and hair can help avoid poisoning incidences. Likewise, deficiency problems can be rapidly resolved with frequent monitoring and supplementation.

## LARKSPURS (*DELPHINIUM* SPP.)

There are more than 80 wild species of larkspurs in North America, and there are a larger number of domestic horticultural varieties. Wild larkspurs are classified into three general categories based primarily on mature plant height and distribution: low, tall, and plains larkspurs (Figure 77.2). The dominant larkspur species in the western United States are shown in Table 77.2. The larkspurs are a major cause of cattle losses on western ranges. As early as 1913, C.D. Marsh reported that



FIGURE 77.2 Low larkspur (*Delphinium bicolor*) is on the left and tall larkspur (*D. barbeyi*) is on the right.

TABLE 77.2 Characteristics of the dominant larkspur species in western North America

Class/species	Height at maturity (cm)	Elevation (m)	Associated plant communities	Toxicity ranking <sup>a</sup>	Typical risk of losses <sup>b</sup>
<b>Tall larkspurs</b>					
<i>D. glaucum</i> <sup>c</sup>	90–200	>2000	Aspen, conifers, alpine meadows	1	Low
<i>D. barbeyi</i>	90–180	>2200	Aspen, conifers, alpine meadows, mountain brush, alpine tundra	2	Moderate to severe
<i>D. glaucescens</i>	76–90	>2000	Mountain meadows, sagebrush	3	Low to moderate
<i>D. occidentale</i>	90–180	>2000	Mountain brush, sagebrush, conifer, aspen	4	Low to severe
<b>Low larkspurs</b>					
<i>D. nuttallianum</i>	20–60	>1200	Mountain brush, sagebrush, aspen, conifer, mountain and foothill meadows	2	–
<i>D. bicolor</i>	20–40	>800	Mountain brush, sagebrush	–	–
<i>D. andersonii</i>	10–60	>1200	Desert shrub, mountain brush, sagebrush, pinyon–juniper	1	–
<b>Plains larkspur</b>					
<i>D. geyeri</i>	40–80	>1500	Desert shrub, mountain brush, sagebrush, short-grass prairie	–	–

<sup>a</sup>Tall larkspur and low larkspur species are each ranked according to the concentration of toxic alkaloids, from greatest (1) to least (4).

<sup>b</sup>The risk of losing cattle to tall larkspur species is a subjective evaluation based on plant toxicity, numbers of grazing cattle threatened during the growing season, and the geographical distribution of the larkspur species. *Delphinium glaucescens* is relatively more toxic late in the growing season compared to mature plants of the other tall larkspur species. Spatial and temporal variability in plant density and toxicity preclude making such a risk assessment ranking for low larkspurs and plains larkspur.

<sup>c</sup>*D. glaucum* = *D. brownii* in Canada; livestock losses to *D. brownii* in Canada may greatly exceed those of *D. glaucum* in the United States.

more cattle deaths on western ranges are caused by *Delphinium* spp. than by any other poisonous plant except locoweed.

## Description

Most of the wild larkspurs have flowers of blue or purple, bilaterally symmetrical, in erect terminal racemes or panicles: five sepals, the upper prolonged backwards in a prominent spur; petals smaller and variable among species, usually four and sometimes two, with the upper pair projecting back inside the spur; and carpals one to five, sometimes fused, ripening into many-seeded follicles.

The tall larkspurs (Figure 77.2) are 1 or 2 m tall or more, flower in summer, and set seed in late summer, subsequently senescing in the fall. In the west, the tall larkspurs typically inhabit higher mountain elevations in the more open moist areas of canyons, draws, and meadows. *Delphiniums* are frequently confused with *Aconitum*, but certain features distinguish them. As implied by the common names, *Delphiniums* have a distinct spur (hence larkspur), whereas *Aconitum* has no spur but a hood (hence monkshood). Tall larkspurs have hollow stems, and their leaves are relatively long petioled, whereas monkshood has stems that are solid and pithy and leaves are short petioled.

Low larkspurs (Figure 77.2) appear similar to tall larkspurs except they grow in different habitats and

locations – that is, lower elevations in drier habitats, on foothills and flats. They appear first as a rosette-like clump in early spring, soon producing an erect flowering stem, usually not more than 1 m in height, and then die back in early summer.

Plains larkspur falls between the low and tall larkspur classifications. Plants range from 0.3 to 2 m tall. It grows on the short-grass plains of Wyoming into Nebraska and in the sagebrush and juniper woodlands of the Colorado Plateau.

## Distribution and habitat

### Low larkspur

*D. nelsonii* – Idaho, South Dakota, Wyoming, Colorado, and Utah

*D. bicolor* – North Dakota, Montana, Wyoming, Oregon, and Washington

*D. andersonii* – Oregon, California, Nevada, Utah, and Idaho

*D. tricornis* – Nebraska and Oklahoma eastward

*D. virescens* – Great Plains, east of the Rockies

### Intermediate larkspur

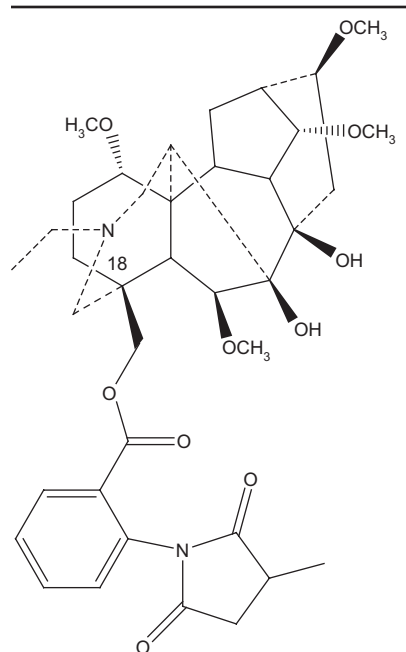
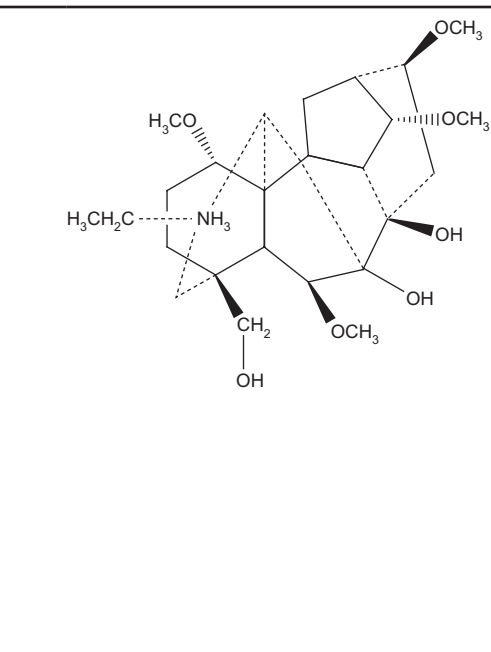
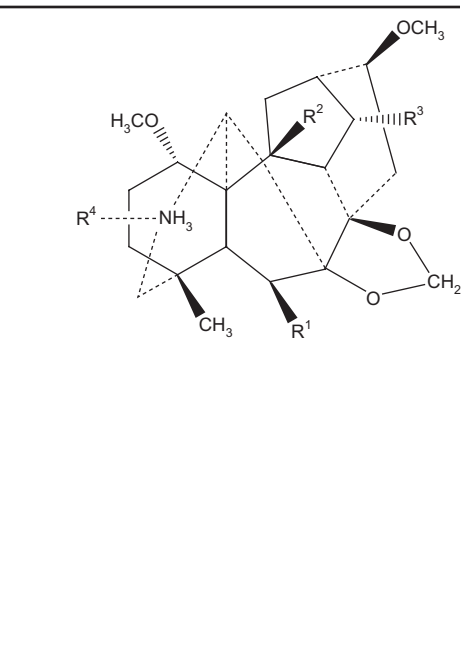
*D. geyeri* – Colorado, Wyoming, Nebraska, and Utah

### Tall larkspur

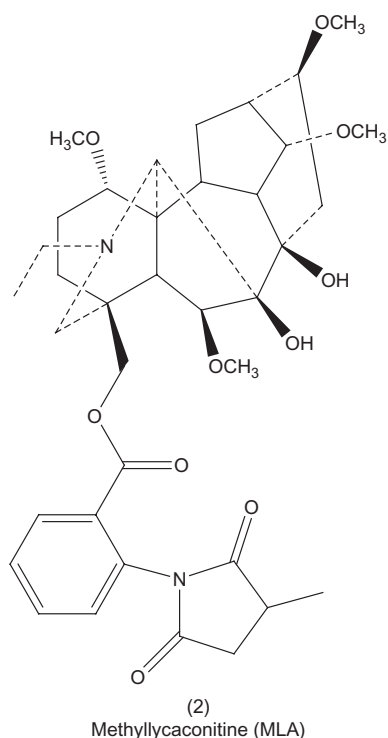
*D. barbeyi* – Utah, Wyoming, Colorado, and New Mexico

*D. occidentale* – Washington, Idaho, Wyoming, Nevada, Utah, and Colorado

TABLE 77.3 Relative toxicity of individual larkspur alkaloids in order of toxicity according to alkaloid class and determined by mouse bioassay

		
<p>MSAL class</p> <p>Alkaloid; LD<sub>50</sub> (mg/kg)</p> <p>Nudicauline; 2.5 i.v.</p> <p>14-Deacetylnudicauline; 4.5 i.v.</p> <p>Methyllycaconitine (MLA); 7.5 i.v.</p> <p>Barbiniine; &gt;115.5 i.v.</p>	<p>Lycoctonine class</p> <p>Alkaloid; LD<sub>50</sub> (mg/kg)</p> <p>Anthranyllycoctonine; &lt;365 s.c.</p> <p>Lycoctonine; 75 i.v.</p> <p>14-Dehydrobrowneine; &gt;254 s.c.</p> <p>Browneine; &gt;720 s.c.</p>	<p>MDL class</p> <p>Alkaloid; LD<sub>50</sub> (mg/kg)</p> <p>Deltaline; 720 s.c., 133 i.v.</p> <p>14-O-acetyldictyocarpine; 110 i.v.</p> <p>Dictyocarpine; &gt;2000 s.c.</p>

i.v., intravenous; MDL, 7,8-methylene dioxyllycoctonine (least toxic); MSAL, methylsuccinimido anthranoyllycoctonine (most toxic); s.c., sub-cutaneous. From Panter *et al.* (2002) and Welch *et al.* (2008).



*D. glaucescens* – Idaho and Montana  
*D. glaucum* – Washington, Oregon, California, Nevada, Idaho, and Montana  
*D. trolliifolium* – Washington, Oregon, and California  
*D. robustum* – Colorado and New Mexico

## Toxicology

Larkspurs (*Delphinium* spp.) are a serious toxic problem for cattle on foothill and mountain rangelands in western North America. The toxicity of larkspur plants is due to norditerpenoid alkaloids, which occur as one of two chemical structural types – the 7,8-methylenedioxylycoctonine (MDL) type and the *N*-(methylsuccinimido) anthranoyllycoctonine (MSAL) type. Although the MSAL-type alkaloids are much more toxic (typically >20×) (Panter *et al.*, 2002), the MDL-type alkaloids are generally more abundant in *D. barbeyi* and *D. occidentale* populations (Gardner *et al.*, 2002). Three MSAL-type alkaloids that are of primary concern are methyllycaconitine (MLA (2)), 14-deacetylnudicauline (DAN), and nudicauline (NUD). MLA and DAN occur



to some extent in all classes of larkspurs, whereas NUD occurs only in low and plains larkspurs. The LD<sub>50</sub> for NUD, MLA, and DAN in mice is 2.7, 4.8, and 4.0 mg/kg intravenously (i.v.), respectively (Table 77.3; Panter *et al.*, 2002). The toxic alkaloid concentration of the tall larkspur (*D. barbeyi*) dosed to cattle in a lethality study was not determined. However, data from a pen study in which Hereford cattle were dosed via oral gavage with ground plant material indicate that a lethal dose of *D. glaucum* is approximately 2.5 g dried plant material/kg body weight (BW) (unpublished observations). The lethal dose in a grazing situation, however, may be quite different due to the fact that tall larkspur is often ingested repeatedly over 2–4 days.

There are two primary structural features necessary for toxicity: (1) an *N*-ethyl bicyclo tertiary alkaloid nitrogen atom and (2) a C-18 anthranilic acid ester. Other studies have also shown that MLA's aromatic ester function is a significant haptophore and that the succinimide group imparts significant toxicity to alkaloids. Two other structural features also enhance toxicity: (1) functionality at the anthranilic acid amine nitrogen and (2) functionality at C-14 (Panter *et al.*, 2002).

Tall larkspur species vary substantially in toxicity, with a relative ranking (most to least toxic, based on the MSAL alkaloid content) of *D. glaucum* (*D. brownii* in Canada), *D. barbeyi*, *D. glaucescens*, and *D. occidentale*. Generally in tall larkspurs, the concentration of MLA and DAN is highest in immature plant tissue. MLA concentrations in immature tall larkspurs may exceed 20 mg/g. Before shattering, tall larkspur pods are relatively high in toxicity (MLA + DAN = 7–12 mg/g). Toxicity declines rapidly in tall larkspurs once pods begin to shatter. Measuring plant toxicity early in the growing season may allow prediction of season-long toxicity and risk (Ralphs *et al.*, 2002).

Due to the fact that the MSAL-type alkaloids are much more toxic than the MDL-type alkaloids, management recommendations for grazing cattle on larkspur-containing ranges are based primarily on the concentration of MSAL-type alkaloids in larkspur (Pfister *et al.*, 2002; Ralphs *et al.*, 2002). However, in many species of tall larkspur, the MDL-type alkaloids are generally more abundant (Pfister *et al.*, 1999; Gardner *et al.*, 2002). Research using a mouse model suggested that MDL-type alkaloids enhance the overall acute toxicity of MLA in an additive manner (Welch *et al.*, 2008). In subsequent studies with cattle, the MSAL-type alkaloids such as MLA were the primary factors responsible for the toxicity of larkspur plants (Welch *et al.*, 2010). However, populations of larkspur plants that contained large amounts of MDL-type alkaloids, in addition to high MSAL-type alkaloid content, were found to be more dangerous to cattle than plants with only high MSAL-type alkaloids.

Consequently, for a larkspur plant to be toxic to livestock, a sufficient quantity of MSAL-type alkaloids is required. However, MDL-type alkaloids appear to potentiate the overall toxicity of the MSAL-type alkaloids and should be considered when predicting potential toxicity of larkspur populations. Therefore, when chemical analyses are performed on larkspur plants to assess their toxic potential, the concentration of both the MSAL-type and the total alkaloids should be determined, with more weight given to the MSAL-type alkaloids.

MLA and NUD are the dominant toxic alkaloids in low larkspurs, with concentration ranges of 0.8–4.5 and 1–4 mg/g, respectively, for MLA and NUD in low larkspur populations (*D. nuttallianum* and *D. andersonii*) in Utah, Colorado, and Arizona (Gardner, unpublished data). Reports of very high concentrations of MLA (up to 8.7 mg/g) in vegetative low larkspur (*D. nuttallianum*) from Canada were contrasted with a concentration of 2 mg/g in flowering plants (Bai *et al.*, 1994). Unlike tall larkspurs, concentrations of toxic alkaloids in low larkspurs do not decline precipitously after senescence (Gardner, unpublished data). MLA is the major toxic alkaloid in plains larkspur (*D. geyeri*), with concentrations ranging from 1 to 4 mg/g in Wyoming and Colorado. We emphasize that NUD is more toxic than MLA, and both alkaloids are frequently found together in low larkspurs (Gardner, unpublished data). Thus, the potential lethality of low larkspurs can be highly variable depending on alkaloid concentrations and plant density.

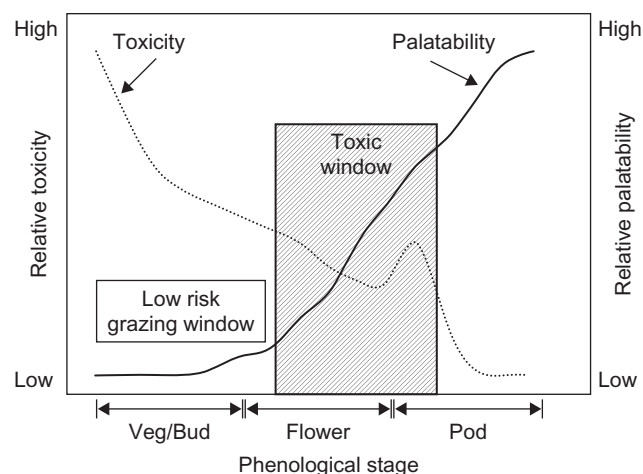
The primary result of larkspur toxicosis is neuromuscular paralysis from blockage at the postsynaptic neuromuscular junction (Benn and Jacyno, 1983). MLA also elicits central effects in mice and rats (Stegelmeier *et al.*, 1998). Larkspur alkaloids compete as postsynaptic inhibitors of acetylcholine particularly at  $\alpha_1$  nicotinic sites (Dobelis *et al.*, 1999). MLA strongly competes with  $\alpha$ -bungarotoxin at nicotinic acetylcholine receptors (nAChRs). Larkspur alkaloid binding to nAChRs appears to be correlated with toxicity in various tissues and may explain sheep tolerance to larkspur if larkspur toxins bind less avidly to nAChRs in sheep (Stegelmeier *et al.*, 1998).

Clinical signs of intoxication include muscular weakness and trembling, straddled stance, periodic collapse into sternal recumbency, respiratory difficulty, and death while in lateral recumbency. An effective dose of larkspur causes labored breathing, rapid and irregular heartbeat, and collapse but not death. The effective i.v. dose for purified MLA is 2 mg/kg BW in calves versus 10 mg/kg in sheep given a single injection (Panter, unpublished data). Nation *et al.* (1982) reported that an effective dose of MLA in cattle receiving two i.v.

injections was 1.1 mg/kg. Conversely, the effective i.v. dose for deltaline in both calves and sheep is 50 mg/kg (Panter, unpublished data). Cattle typically show clinical signs (i.e., tremors and periodic collapse) when given an MLA + DAN dose (i.e., via ground plant) of  $20 \pm 3$  mg/kg BW (Pfister *et al.*, 1994). Stress and/or exertion will reduce the effective dose. Assuming an MLA + DAN concentration of 5 mg/g (dry wt), a 450-kg cow may show clinical signs after rapidly eating 1.8 kg (dry wt) of tall larkspur ( $\approx 7.2$  kg wet wt).

Key factors in larkspur intoxication are the amount eaten and the rate of ingestion. Studies utilizing direct observations of grazing animals have provided a number of insights into the amount and timing of tall larkspur ingestion. More than 10 such studies have been conducted since 1986 and have led to two major conclusions: (1) cattle eat little or no tall larkspur before the plant has elongated flowering racemes, and (2) weather patterns are very important determinants of larkspur consumption (Pfister *et al.*, 1999). Cattle often eat more tall larkspur during summer storms and reduce larkspur consumption during drought for reasons that are not clear. Another variable that may play a role in the susceptibility of cattle to larkspur toxicosis is breed differences. Anecdotal observations of differences in the susceptibility of different breeds of cattle to larkspur toxicosis have been supported using a rodent model in which different strains of mice had up to a twofold difference in LD<sub>50</sub> to MLA (Welch *et al.*, 2009a).

Cattle generally begin consuming tall larkspur after flowering racemes are elongated, and consumption increases as larkspur matures. Consumption usually peaks during the pod stage of growth in late summer, when cattle may eat large quantities (25–30% of diet as herd average; >60% on some days by individual animals). Because larkspur toxicity generally declines throughout the growing season and cattle tend to eat more larkspur after flowering, the period of greatest danger has been termed a “toxic window” (Pfister *et al.*, 2002). This toxic window extends from the flower stage into the pod stage, or approximately 5 weeks depending on temperature and elevation (Figure 77.3). Many ranchers typically defer grazing on tall larkspur-infested ranges until the flower stage to avoid death losses. This approach wastes much valuable forage and often places cattle into larkspur-infested pastures when risk of losses is high. An additional 4–6 weeks of grazing may be obtained by grazing these ranges early, before larkspur elongates flowering racemes. The risk of losing cattle is low when grazing before flowering, even though larkspur is very toxic, because larkspur consumption is typically very low. Once pods are mature and begin to shatter, larkspur ranges can usually be grazed with impunity because pod toxicity declines rapidly, and leaf



**FIGURE 77.3** Relationship between toxicity and palatability in tall larkspurs. Most cattle deaths are predicted to occur during the toxic window when the concentration of toxic alkaloids is relatively high and consumption by cattle begins to increase after flowering racemes elongate. There is a low-risk grazing window early in the season before flowering when larkspur is generally very toxic but risk is low because cattle typically eat little tall larkspur during this phenological stage. There is also a low-risk grazing window in late summer after pods shatter.

toxicity is low. Based on limited study, cattle increase consumption of low larkspur after flowering, and increases in grazing pressure increase amounts of low larkspur eaten by cattle. No consistent consumption patterns of plains larkspur (*D. geyeri*) by cattle were found; thus, management recommendations need to be tailored to each specific year and location (Pfister *et al.*, 2002).

Studies have been performed to characterize the kinetic profile of larkspur alkaloids in cattle (Green *et al.*, 2009b, 2011). In these studies, the elimination half-life of MLA from cattle dosed once with dried and finely ground larkspur via oral gavage was approximately 20 h. In addition, the  $T_{max}$ , or time to maximal toxin concentration, was approximately 10 h. These data suggest that cattle that have consumed larkspur will be most susceptible to poisoning 10 h after consumption, and that after the cattle have stopped consuming larkspur, greater than 99% of the toxins will be eliminated after 6 days.

## Prevention and management of poisoning

### Grazing management

A simple and low-risk grazing management scheme can often be used based simply on tall larkspur growth and phenology: (1) graze during early summer when sufficient forage is available until larkspur elongates flowering racemes (4–6 weeks depending on elevation and

weather); (2) remove livestock, or contend with potentially high risk from flowering to early pod stages of growth (4 or 5 weeks); and (3) graze with low risk during the late season when larkspur pods begin to shatter (4–6 weeks). This scheme can be refined substantially if livestock producers periodically obtain an estimate of the toxicity of tall larkspur, and if ranchers spend time periodically observing and documenting larkspur consumption by grazing cattle.

Management to reduce losses to low larkspur begins with recognition of the plant during spring. Vegetative low larkspur plants will typically begin growth before the major forage grasses. Low larkspur populations fluctuate with environmental conditions (Pfister, unpublished data). Risk of losing cattle is much higher during years with dense populations. During those years, recognizing the plant, and finding alternative pasture or waiting to graze infested pastures for 4–6 weeks until the low larkspur has dried up, will reduce losses. In addition, it is recommended that animals not be watered or provided mineral supplementation in areas that have high densities of larkspurs.

#### *Graze sheep before cattle*

Sheep can be herded into or bedded on the patches to reduce larkspur availability or acceptability to cattle on tall larkspur-infested ranges where larkspur grows as discrete patches. In those areas in which larkspur is uniformly spaced over a pasture, sheep must eat immature larkspur and leave sufficient feed for subsequent grazing by cattle. This can be problematic because early growth tall larkspur may not be palatable to sheep. Our observations (Pfister, unpublished data) indicate that sheep eat little low larkspur (*D. nuttallianum*) unless a high stock density is used.

#### *Drug intervention*

A variety of remedies have been applied in the field when ranchers find intoxicated animals (e.g., bleeding by cutting the tail), but most are without a solid scientific rationale. Any imagined success with these treatments was probably related to the dose. If less than a lethal dose were ingested, the animal would likely recover despite any treatment, unless bloat or aspiration pneumonia occurred during recumbency. Treatment for overt poisoning is usually symptomatic, and recovery is often spontaneous if animals are not stressed further by driving. Once the animal is observed showing muscular tremors, it should be allowed to drop back and proceed at its own pace. Poisoned animals should never be forced to continue moving because this will exacerbate the clinical effects and can result in death. Drugs that increase acetylcholine effectiveness at the neuromuscular

junction have potential for reversing larkspur toxicosis or reducing susceptibility. The cholinergic drug physostigmine (0.08 mg/kg i.v.) has been successfully used under field and pen conditions to reverse clinical larkspur intoxication (Nation *et al.*, 1982; Pfister *et al.*, 1994). Similarly, i.v. administration of neostigmine (0.04 mg/kg) significantly reduced clinical signs in cattle (Green *et al.*, 2009a), and neostigmine administered intramuscularly at 0.02 mg/kg can be used as a rescue treatment for cattle in recumbency. This reversal lasts approximately 2 h, and repeated injections of physostigmine are sometimes required. Under field conditions, physostigmine temporarily abates clinical signs and animals quickly (~15 min) become ambulatory. Depending on the larkspur dose, the intoxication may recur. The use of physostigmine-based treatments may aggravate losses in the absence of further treatment if suddenly ambulatory animals later develop increased muscular fatigue, dyspnea, and death.

#### *Herbicidal control*

Larkspur losses can be greatly reduced if dense larkspur populations are reduced by herbicides. Picloram, metsulfuron, and glyphosate have proven to be effective in killing tall larkspurs when applied at specific growth stages (Ralphs *et al.*, 1991). These herbicides do not reduce toxic alkaloid concentrations in treated larkspur plants, and metsulfuron may increase toxicity. Therefore, sprayed areas should not be grazed until the following growing season.

## LUPINES (*LUPINUS* SPP.)

The *Lupinus* genus contains more than 150 species of annual, perennial, or soft woody shrub lupines. More than 95 species occur in California alone. The lupines are rich in alkaloids, responsible for most of the toxic and teratogenic properties. There are domestic lupines that through plant breeding are low in alkaloid content and have been selected for ornamental purposes or for animal and human food. Only those range lupines known to cause poisoning or birth defects are discussed here.

Stockmen have long recognized the toxicity of lupines when livestock, particularly sheep, were poisoned in the fall by the pods and seeds of lupine. Major losses in sheep were reported in the 1950s, and individual flock losses of hundreds and even thousands were reported. Lupines are also poisonous to other livestock, and field cases of poisoning in cattle, horses, and goats have been reported. However, the most recognized condition of lupine ingestion is the “crooked calf syndrome,” a congenital condition in calves resulting in skeletal contracture-type malformations and cleft palate after their



FIGURE 77.4 *Lupinus leucophyllus*.

mothers have grazed lupines during sensitive periods of pregnancy (Panter *et al.*, 1999a,b). The condition was first reported in 1959 and experimentally confirmed after large outbreaks in Oregon and Montana in 1967.

## Description

Lupines belong to the Leguminosae family, with alternate palmately compound leaves with 5–17 oblong to lanceolate leaflets (Figure 77.4). Flowers are terminal legume-like and can be blue, purple, white, yellow, or reddish. The seeds are flattened in legume-like pods. Range lupines are generally low, perennial or annual forbs, and species and varieties are taxonomically difficult because extensive hybridization occurs and the literature may be somewhat confusing in this regard.

The plant grows early in spring, flowering in early to mid-summer, and forms pods in late summer or early fall. Seedlings may germinate in fall if temperature and moisture are conducive for seed germination. Some lupines are annuals and others are woody and shrub-like; however, neither the annual nor the woody species have been reported in livestock poisoning.

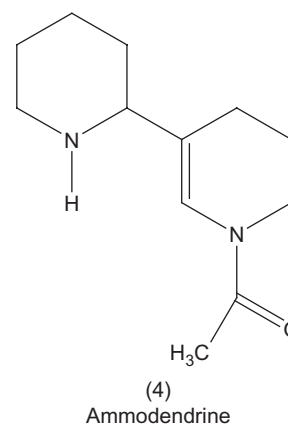
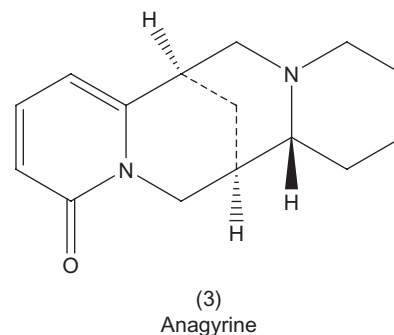
Chemical profiles (chemo-taxonomy) can support or contradict taxonomic identification, and this is a valuable resource if included in a voucher specimen, especially if the identified species is implicated in toxicoses or teratogenicity and is identified by geographical location. Cook *et al.* (2009b) demonstrated this variability in a single species, *Lupinus sulphureus*, which is yellow lupine species, selected from herbaria or collected from different geographical regions of Oregon, Washington, and British Columbia. A total of seven distinct alkaloid

profiles were reported, and each alkaloid profile was unique in its geographical distribution and its potential risk to livestock. A collection near Pendleton, Oregon, came from a population that was responsible for a significant outbreak of crooked calf syndrome in a herd of cattle (56% loss) and the alkaloid profile showed high levels of the teratogen anagyrene (Panter *et al.*, 1997). Therefore, taxonomic classification without chemical support is of little value in predicting risk of crooked calf syndrome.

## Distribution

Most species of lupines grow in states and provinces from the Rocky Mountains westward. They are classified as increaser species; that is, they increase in abundance following disturbance from overgrazing or fire, to the point that they may dominate the plant community at times. Their populations also cycle, increasing in wet years and dying back during drought. Table 77.4 lists species involved in toxicity and their common names, habitats, and geographical distribution.

## Toxicology



Most lupine species contain quinolizidine alkaloids, a few contain piperidine alkaloids, and some contain both. The specific alkaloids responsible for crooked calf syndrome are anagyrene (3), ammodendrine (4), and



TABLE 77.4 Lupine species known to be toxic or teratogenic

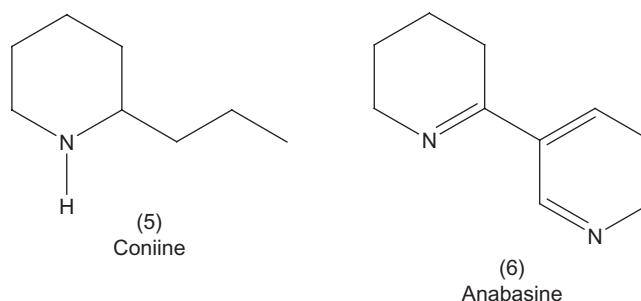
Scientific name	Common name	Habitat	Distribution
<i>L. alpestris</i>	Mountain silvery lupine	Dry rocky soils	CA to MT, CO and AZ
<i>L. arcticus</i>			AK, British Columbia
<i>L. andersonii</i>	Anderson's lupine	Mountain meadows and clearings	Sierra Nevada, CA to 2900 m elevation
<i>L. arbustus</i>	Spur lupine	Dry open hillsides	NV, CA to CO, western MT
<i>L. argenteus</i>	Silvery lupine	1200–1500 m elevation; dry flats, slopes, woods, open hillsides	CA to MT, NM, OR, ID, UT, NV
<i>L. caudatus</i>	Tail cup lupine		CA, OR, ID to UT, NV
<i>L. cyaneus</i>			MT
<i>L. formosus</i>	Lunara lupine	Dry flats	CA
<i>L. greenei</i>		Plains and hills	WY, NV, CO, AZ
<i>L. latifolius</i>	Broad-leaved lupine	Mountain meadows, stream banks	Pacific states
<i>L. laxiflorus</i>	Spurred lupine	Hillsides, dry soils	WA, OR, ID
<i>L. leucophyllus</i>	White-leaved lupine, poison lupine	Dry soils	WA, MT, UT, CA
<i>L. nootkatensis</i>	Nootka lupine		AK
<i>L. onustus</i>	Plumes lupine	Open pine wood	CA
<i>L. polyphyllus</i> (five varieties)	Meadow lupine	Mountain meadows, banks	WA, British Columbia
<i>L. pusillus</i>	Rusty lupine, small lupine	Dry plains	British Columbia to KS, NM, AZ
<i>L. sericeus</i>	Silky lupine	Dry hills, valleys	UT, WY to MT, British Columbia
<i>L. sulphureus</i>	Yellow lupine	Open hillsides	OR

N-methyl ammodendrine. Hence, risk is based on chemical profile and the presence and concentration of these teratogenic alkaloids. It is known that chemical profile and concentration differ, resulting in changing levels of toxicity within and between species and populations. The chemical phenology has been studied in *L. caudatus* and *L. leucophyllus* (Lee *et al.*, 2007b). Total alkaloid concentration is high in the new early growth but diluted as the plant biomass increases. Pools of total alkaloids increase during the phenological growth stages and peak at the pod stage, concentrating in the pods. The teratogenic alkaloid anagryne appears to be an end product in the biosynthetic pathway and accumulates in the floral parts and is stored in the seed. Following seed shatter, both concentration and pools of all alkaloids decline precipitously, leaving the senescent plant relatively nontoxic.

Stockmen recognize the toxicity of lupines in the fall when the pods and seeds are ripe. Historically, lupines were responsible for more sheep deaths than any other single plant in Montana, Idaho, and Utah. Most losses occurred from hungry sheep grazing seed pods. Poisoning occurred following trucking or trailing bands in late summer or fall or after getting caught in early snowstorms that covered herbaceous vegetation. Hungry sheep nonselectively grazed lupine pods, which are highest in alkaloids, and were poisoned. Large losses have also occurred when lupine hay harvested in the seed pod stage was fed in winter. Seeds alone

can be toxic to sheep at 0.25–1.5% of their body weight depending on alkaloid composition. A few cases of poisoning occurred on young plants. Losses of 80–100 sheep in multiple bands have been reported during the past 5 years in Idaho and Wyoming (Panter, personal communication, 2005).

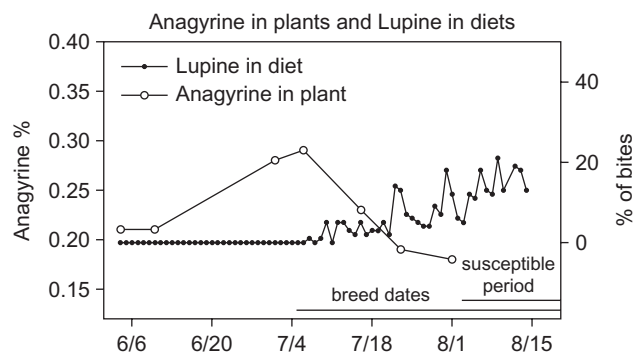
Poisoning by lupine plants should not be confused with lupinosis reported in Australia. This condition is entirely different and is a mycotoxicosis of livestock caused by toxins produced by the fungus *Phomopsis leptostromiformis*, which colonizes domestic lupine stubble. It affects livestock that graze lupine stubble and limits the use of this animal feed in Australia.



The lupine-induced crooked calf syndrome was first reported in 1959 and 1960 and experimentally confirmed in 1967 (Panter *et al.*, 1999a). Crooked calf disease includes various skeletal contracture-type birth defects and occasionally cleft palate. The skeletal defects

are similar to an inherited genetic condition reported in Charolais cattle. Based on epidemiologic evidence and chemical comparison of teratogenic and nonteratogenic lupines, the quinolizidine alkaloid anagryne was determined to be the teratogen (Keeler, 1973). A second teratogen, a piperidine alkaloid called ammodendrine found in *Lupinus formosus*, was also demonstrated to cause the condition (Keeler and Panter, 1989). Further research determined that the anagryne-containing lupines only caused birth defects in cattle and did not affect sheep or goats; however, the piperidine-containing lupine *L. formosus* induced similar birth defects in cattle and goats (Keeler and Panter, 1989). This led to interesting speculation about possible metabolism or absorption differences between cattle and small ruminants. Keeler and Panter (1989) hypothesized that perhaps the cow was metabolizing the anagryne to a complex piperidine, meeting the structural characteristics determined for a teratogenic piperidine. This was supported by feeding trials with other piperidine-containing plants, extracts, and pure compounds. Coniine (5), a simple piperidine from poison hemlock, and anabasine (6), a simple piperidine from tree tobacco (*Nicotiana glauca*), induced the same defects in cattle, sheep, and goats. Although comparative studies support the hypothesis that the cow may metabolize the quinolizidine anagryne to a complex piperidine, evidence regarding the absorption and elimination patterns of many of the quinolizidine alkaloids, including anagryne, in cattle, sheep, and goats does not support the metabolism theory (Gardner and Panter, 1993). This research is currently ongoing at the Poisonous Plant Research Laboratory. A review of lupine-induced cases of crooked calf syndrome reported to the Poisonous Plant Research Lab during the past 20 years can be found in Lee *et al.* (2007a).

Different lupines produce varying toxic syndromes in a given species of livestock, apparently because the alkaloid profile varies remarkably among species. Season and environment influence alkaloid concentration in a given species of lupine. Generally, alkaloid content is highest in young plants and in mature seeds. Alkaloids are not lost upon drying, so wild hay may be highly toxic if young lupine plants or especially seed pods are present. For many lupines, the time and degree of seeding vary from year to year. Most losses occur under conditions in which animals consume large amounts of pods in a brief period, such as when they are being driven through an area of heavy lupine growth, unloaded into such an area, trailed through an area where the grass is covered by snow but the lupine is not, or when feeding lupine hay when lupine is in the pod stage, which apparently is palatable. Most serious poisonings may occur in the late summer or early fall because lupine remains green after other forage has dried and seed pods are present. Once the poisonings were understood, the



**FIGURE 77.5** The relationship between the concentration of the teratogen anagryne in the plant and the amount of lupine in the diets of cattle during breeding and susceptible gestational stages.

practice of harvesting lupine hay for winter sheep feed was discontinued.

#### Cattle grazing

Lupine is not very palatable to cattle, although it has been considered fair to good quality feed on some ranges that are heavily utilized. Its palatability or acceptability depends on availability and maturity of other forage. In a grazing study of velvet lupine (*L. leucophyllus*) on annual cheatgrass ranges in eastern Washington (Ralphs *et al.*, 2006), cows selected lupine in July and August after cheatgrass dried and other forbs were depleted or matured and became rank. The deep-rooted lupine remained green and succulent longer into the summer than the other forage. Lupine was higher in crude protein and lower in fiber (NDF) than the other forages throughout the season (the crude protein level in foliage was 15%, and in seeds it was 36%). However, the thick, velvety pubescence was believed to be partially responsible for its lack of palatability. The high alkaloid concentration in the floral parts and seed pods may also have contributed to its lack of palatability. Once the seeds shattered in early July, the alkaloid concentration declined, and the cows began selecting lupine. Figure 77.5 shows the overlap of lupine consumption with the susceptible period of gestation, in relation to the anagryne concentration in the lupine plants. Cattle selected velvet lupine in July after cheatgrass and other forbs matured.

The abundance of lupine is another factor influencing the amount of lupine consumed. Lupine population cycles are influenced by weather patterns. Catastrophic losses from lupine-induced crooked calves occurred in the Channel Scabland region of eastern Washington in 1997. Annual precipitation from 1995 to 1997 was 33% above average, initiating an outbreak of lupine throughout the region. The density of velvet lupine plants has

declined since then (Ralphs, unpublished data), and the incidence of crooked calves has returned to what has become an acceptable tolerance of 1–5% incidence.

Clinical signs of poisoning are those of muscular weakness (neuromuscular blockade) beginning with nervousness, frequent urination and defecation, depression, frothing at the mouth, relaxation of the nictitating membrane, ataxia, muscular fasciculations, weakness, lethargy, collapse, sternal recumbency followed by lateral recumbency, respiratory failure, and death. Signs may appear within 15 min to 1 h after ingestion or as late as 24 h depending on the amount and rate of ingestion. Death usually results from respiratory paralysis.

The incidence of crooked calves is variable geographically and from year to year within a given herd. Up to 100% of a given calf crop may be affected, and individuals may be more severely affected than others. Affected calves are generally born alive at full term. Dystocia may occur when calves are severely deformed and assistance is required, often by cesarean section.

Arthrogryposis is the most common malformation observed and is often accompanied by one or more of the following: scoliosis, torticollis, kyphosis, or cleft palate. Elbow joints are often immobile because of malalignment of the ulna with the articular surfaces of the distal extremity of the humerus. The part of the limb distal to the elbow joint is often rotated laterally. In crooked calf disease, the osseous changes observed are permanent and generally become progressively worse as the calf grows and its limbs are subjected to greater load-bearing stress. Frequently, minor contractions such as “buck knees” often attributed to lupine will resolve on their own and the calf will appear relatively normal.

No breed predilection or genetic susceptibility to the lupine-induced condition has been determined. Likewise, lactation or age did not influence the propensity for cattle to graze lupine (Pfister *et al.*, 2008a). However, body condition was shown to affect the absorption and elimination half-life of anagryne in sheep (Lopez-Ortiz *et al.*, 2004). Research on cattle has demonstrated that body condition impacts absorption, distribution, and elimination of the alkaloids and therefore could impact the risk of toxicity or teratogenic outcome of lupines (Lee *et al.*, 2008d). This difference in toxicokinetics was significant for two teratogenic alkaloids – anagryne and ammodendrine. The disposition of teratogenic alkaloids in the fetal compartment is unknown and will require further research.

The sensitive gestational period in the pregnant cow for exposure is 40–70 days with suspicious periods extending to day 100 (Panter *et al.*, 1997). The condition has been experimentally induced with dried ground lupine at 1 g/kg BW and with semi-purified preparations of anagryne (the apparent teratogen) at 30 mg anagryne/kg BW fed daily from 30 to 70 days of

gestation. The dose range of anagryne to cause crooked calves is 6.5–11.9 mg/kg BW/day for 3 or 4 weeks during gestation days 40–70. Crooked calf disease has also been induced by feeding the piperidine alkaloid-containing lupine, *L. formosus* (Keeler and Panter, 1989). The teratogenic piperidines, ammodendrine, *N*-acetylhystrine, and *N*-methyl ammodendrine, are absorbed quickly after ingestion and can be detected in blood plasma by 0.5 h, with peak levels maintained for more than 24 h (Gardner and Panter, 1993). The mechanism of action has been determined to be an alkaloid-induced reduction in fetal movement by a neuromuscular blocking effect during the critical stages of gestation (Panter *et al.*, 1990a). This inhibition of fetal movement is due to stimulation followed by desensitization of skeletal muscle-type nAChR (Lee *et al.*, 2006). This mechanism is a common factor for multiple alkaloids found in many species of lupines, poison hemlock (*Conium*), and wild tree tobacco (*N. glauca*), and research using TE-671 cells that express human fetal muscle-type nAChR and SH-SY5Y cells that express human autonomic-type nAChR supports this mechanism (Green *et al.*, 2010). Interestingly, many of these teratogenic alkaloids are produced in the plants as enantiomeric pairs and exhibit differences in activity using these specialized cells and when compared using a mouse bioassay (Lee *et al.*, 2006, 2008a–c). This inhibited fetal activity is responsible for the skeletal contracture malformations and cleft palates (Panter *et al.*, 1990a), and a goat model developed in the 1990s using anabesine as the teratogen and ultrasound imaging to evaluate fetotoxicity has been utilized for biomedical research to improve treatments and potential fetal intervention for cleft palate in children (Panter and Keeler, 1992; Weinzwieg *et al.*, 1999, 2008).

## Prevention, management, and treatment

Keeler *et al.* (1977) proposed a simple management solution to prevent crooked calves: stagger grazing of lupine-infested pastures so that the susceptible period of gestation (40–70 days) does not overlap the flower and pod stage of growth when anagryne is highest. Ralphs *et al.* (2006) refined Keeler’s recommendations to restrict access during the susceptible period of gestation, when anagryne concentration is still high in the flower and pod stage, only when cattle are likely to eat lupine, and in years when it is abundant. Panter (unpublished data) suggested that intermittent grazing between lupine pastures and clean pastures would allow the fetus to regain normal movement for a few days during the sensitive stage of gestation. It has been hypothesized that inhibited fetal movement over a prolonged period of time is required for severe malformations to occur (Panter *et al.*, 1999a).

Lupines are easily controlled with 2,4-D-type broad-leaf herbicides (Ralphs *et al.*, 1991); however, herbicide treatment alone rarely provides long-term solutions to poisonous plant problems. Seed reserves in the soil will rapidly reestablish the stands if grazing management practices are not implemented.

Death losses in sheep can be reduced by recognizing the variability in lupine toxicity with stage of growth and the conditions under which animals graze the plant. Providing a choice of other quality forages usually prevents excess lupine grazing. The dangerous period of plant growth for sheep exists mainly with plants in the pod stage. The hazard increases if sheep are hungry, as is often the case with crowding, hauling, driving, or overgrazed conditions. The hazard is reduced or eliminated when lupine is in post-seed stage.

Treatment for overt poisoning is usually symptomatic, and recovery is often spontaneous if animals are not stressed further by driving. Once the animal is observed showing muscular tremors, it should be allowed to drop back and proceed at its own pace. Poisoned animals should never be forced to continue moving because this will exacerbate the clinical effects and can result in death. The elimination of the toxic alkaloids in the urine is quite rapid ( $t_{1/2} = 6.32 - 6.88\text{h}$ ) and begins within hours of ingestion (Lopez-Ortiz *et al.*, 2004). Therefore, allowing the animal to rest and move slowly will often result in full recovery within 24h. There is no treatment for the malformations, and euthanasia is recommended for the serious skeletal defects and cleft palate. However, less severe contracture defects, particularly of the front legs (buck knees), will often resolve if the knee joint can be locked within 1 week after birth. If not, the defect generally becomes worse with growth and size, and although the animal will continue to grow, the front legs will break down and the animal will not be adequate for the feedlot.

## Summary

A reduction in incidence can be expected and has been achieved by using one or more of the following: (1) coordinating grazing periods according to plant growth stage, (2) changing time of breeding by either advancing or delaying or changing from spring to fall calving, (3) reducing lupine populations through herbicide treatment, and (4) intermittent grazing between clean pastures and lupine pastures to break the cycle of lupine ingestion.

## POISON HEMLOCK (*CONIUM MACULATUM*)

Poison hemlock was introduced into the United States as an ornamental herb and grows throughout the country.



**FIGURE 77.6** The hemlocks – poison hemlock (*Conium maculatum*) on the left and water hemlock (*Cicuta douglassii*) on the right. Note the tubers and partitioned stalk on water hemlock versus the single taproot on poison hemlock. Leaf structure is different, and poison hemlock has purple spots on the stems.

Generally, poison hemlock grows in waste areas where adequate moisture will sustain the biennial stands. Four species are recognized worldwide, but only one (*Conium maculatum*) is found in the United States. Historically, poison hemlock has been associated with human poisoning more than livestock and is believed to be the tea used to execute Socrates.

## Description

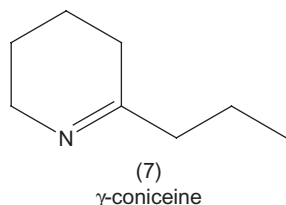
Unlike the lupines, locoweeds, etc., multiple *Conium* species are few worldwide, and only one species, *C. maculatum*, is described in the United States. *Conium maculatum* is a biennial plant 1–2.5m tall (Figure 77.6). The stems are stout, rigid, smooth, and hollow except at the nodes. A distinguishing characteristic of the plant is the purple spots found up and down the main stem and a single carrot-like taproot. The leaves are large, triangular, fern-like, and alternate on the erect stem. The stem grows the second year from a rosette and taproot established from seed. Frequently, seeds will germinate in the fall before winter and then complete their reproductive cycle the following year. The flowers are small, white or cream colored, and in umbellate clusters. The fruits are grayish brown with conspicuous wavy, knotted ridges. The plant has a fleshy, usually unbranched, white taproot that



looks like parsnips. Roots have been mistaken for parsnip, seeds for anise, and leaves for parsley.

The geographical distribution of poison hemlock is throughout the United States, restricted only by cultivation and adequate moisture to sustain stands of the plant from year to year. The plant usually grows in waste places where moisture may accumulate and protected from cultivation.

## Toxicology



Eight piperidine alkaloids are known in poison hemlock, five of which are commonly discussed in the literature. Two alkaloids (coniine (5) and  $\gamma$ -coniceine (7)) are prevalent and likely responsible for toxicity and teratogenicity of the plant.  $\gamma$ -Coniceine is the predominant alkaloid in the early vegetative stage of plant growth and is a biochemical precursor to the other *Conium* alkaloids (Panter and Keeler, 1989). Coniine predominates in late growth and is found mainly in the seeds.  $\gamma$ -Coniceine is seven or eight times more toxic than coniine in mice. This makes the early growth plant most dangerous in the early spring and the seedlings and regrowth again in the fall. This is also the time when green feed is limited to livestock and may impact their propensity to graze this plant. Seeds, which are very toxic, can contaminate poultry and swine cereal grains (Panter and Keeler, 1989). Plants often lose their toxicity upon drying, but seeds remain toxic as long as the seed coat is intact.

An analysis of a single plant of *Conium*, second-year rosette, revealed  $\gamma$ -coniceine levels of 387, 326, 198, 176, and 850 mg/g fresh plant for whole plant, root crown, stem, leaf, and green seed, respectively. Coniine was only detected in the leaf at 12 mg/g fresh plant (Panter and Gardner, unpublished data, 1994).

The clinical signs of toxicity are the same in all species and include initial stimulation (nervousness) resulting in frequent urination and defecation (no diarrhea), rapid pulse, temporarily impaired vision from the nictitating membrane covering the eyes, muscular weakness, muscle fasciculations, ataxia, incoordination followed by depression, recumbency, collapse, and death from respiratory failure (Panter *et al.*, 1988).

*Conium* plant and seed are teratogenic, causing contracture-type skeletal defects and cleft palate like those of lupine. Field cases of teratogenesis have been reported

in cattle and swine and experimentally induced in cattle, swine, sheep, and goats (Panter *et al.*, 1999a). Birth defects include arthrogryposis (twisting of front legs), scoliosis (deviation of spine), torticollis (twisted neck), and cleft palate. Field cases of skeletal defects and cleft palate in swine and cattle have been confirmed experimentally.

In cattle, the susceptible period for *Conium*-induced terata is the same as that described for lupine and is between day 40 and day 70 of gestation. The defects, susceptible period of pregnancy, and probable mechanism of action are the same as those of crooked calf disease induced by lupines (Panter *et al.*, 1999a). In brief, these alkaloids and their enantiomers in poison hemlock, lupines, and *N. glauca* were more effective in depolarizing the specialized cells TE-671, which express human fetal muscle-type nAChR, relative to SH-SY5Y, which predominantly express autonomic nAChRs, in a structure-activity relationship (Panter *et al.*, 1990a; Lee *et al.*, 2006, 2008b; Green *et al.*, 2010). In swine, sheep, and goats, the susceptible period of gestation is 30–60 days. Cleft palate has been induced in goats only when plant or toxins were fed from 35 to 41 days of gestation (Panter and Keeler, 1992).

Field cases of poisoning have been reported in cattle, swine, horses, goats, elk, turkeys, quail, chickens, and Canadian geese (Panter *et al.*, 1999a). Poisoning in wild geese eating small seedlings in early spring was most recently reported (Panter, personal communication). Human cases of poisoning are frequently reported in the literature, and a case of a child and his father mistakenly ingesting the plant has been reported. Field cases of teratogenesis have been reported in cattle and swine and experimentally induced in cattle, sheep, goats, and swine (Panter *et al.*, 1990a). Pigs become habituated to poison hemlock, and if access to the plant is not limited, they will eat lethal amounts within a short time.

There are no diagnostic lesions in poisoned animals, and diagnosis is based on clinical history of exposure and/or alkaloid detection in liver, urine, or blood. At necropsy, the presence of plant in the stomach and a characteristic pungent odor in the contents with chemical confirmation of the alkaloids may be diagnostic.

## Prevention and treatment

Prevention of poisoning is based on recognizing the plant and its toxicity and avoidance of livestock exposure when hungry. If a lethal dose has not been ingested, the clinical signs will pass spontaneously, and a full recovery can be expected. Avoidance of stressing animals poisoned on *Conium* is recommended. However, if lethal doses have been ingested, supporting respiration,

gastric lavage, and activated charcoal are recommended. Control of plants is easily accomplished using broadleaf herbicides; however, persistent control measures are recommended because seed reserves in the soil will quickly reestablish a population.

The mechanism of action of the *Conium* alkaloids is twofold. The most serious effect occurs at the neuromuscular junction, where they act as nondepolarizing blockers like curare. Systemically, the toxins cause biphasic nicotinic effects, including salivation, mydriasis, and tachycardia, followed by bradycardia as a result of their action at the autonomic ganglia. The teratogenic effects are undoubtedly related to the neuromuscular effects on the fetus and have been shown to be related to reduction in fetal movement (Panter *et al.*, 1990a). Likewise, cleft palate is caused by the tongue interfering in palate closure during the reduced fetal movement and occurs during days 30–40 of gestation in swine, 32–41 days in goats, and 40–50 days in cattle (Panter and Keeler, 1992).

## WATER HEMLOCK (*CICUTA* SPP.)

Water hemlock (*Cicuta* spp.) is among the most violently poisonous plants known to humans. It is often confused with poison hemlock because of its name, growth patterns, and appearance. There are distinct differences in appearance, as shown in Figure 77.6.

### Distribution

There are approximately 20 species of *Cicuta* throughout the world, and all are poisonous. Most of these species are found in North America; nine are common in the United States, and seven are found in the western United States (Table 77.5).

### Description

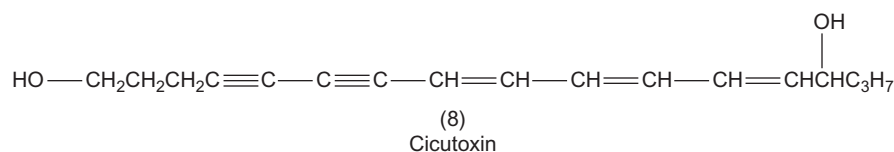
Water hemlock is often confused with poison hemlock; in fact, there are similar plant characteristics and both belong to the Umbelliferae family (Figure 77.6). However, their toxic effects are dramatically different,

and when toxicoses occur, differentiation between the two genera is important.

Water hemlock (*Cicuta*) is a biennial, 1 or 2 m tall, with thickened tubers possessing long, slender parsnip-like roots radiating out from the main tuber. Water hemlock frequently grows directly in streambeds or water sources and is limited to wet habitats. The tubers are bulbous and exhibit several chambers or cavities separated by cross-partitions as seen in a vertical cut through the tuber at the base of the stem. The cut surface of the stem or tuber exudes a yellowish, thick, oily liquid believed to be predominantly cicutoxin (8). This oily substance has a parsnip-like odor. The chambered tuber is a distinguishing feature of this plant (Figure 77.6). The roots extending from the tuber may be solid, white, and fleshy, closely resembling a parsnip. The stems are hollow except at the nodes, hairless, and occasionally have purple stripes up the stem. Leaf stalks alternate up the stem and leaves two or three pinnately divided may be 30–60 cm long. Flowers are small, white or cream colored, and in terminal umbrella-shaped clusters. The fruits are small, with prominent ribs, and encased in a hard brownish shell very similar to those of poison hemlock. The primary distinguishing feature separating water and poison hemlock is the tuberous roots with the very distinct partitions in water hemlock (Figure 77.6). Poison hemlock lacks the large tubers, although occasionally there are very small partitions observed in the area where the root and stem connect if the plant is cut vertically. The parsnip-like root is a single taproot in poison hemlock, whereas there are often multiple white parsnip-like roots radiating off the main tubers in water hemlock (Figure 77.6).

TABLE 77.5 Water hemlock species and geographical distribution

Species	Geographical distribution
<i>C. bulbifera</i>	Northern U.S., southern Canada
<i>C. bolanderi</i>	Central to western CA
<i>C. californica</i>	Central to western CA
<i>C. douglasii</i>	Throughout U.S.
<i>C. machenziana</i>	Southeastern AK north to the Brooks Range
<i>C. occidentalis</i>	Rocky Mountains, Black Hills, WA; south into NV and NM
<i>C. vagans</i>	Pacific Northwest U.S. and Canada



## Toxicology

The toxic principle in water hemlock is a long-chain, highly unsaturated alcohol called cicutoxin. Water hemlock acts on the central nervous system as a stimulant, inducing violent grand mal seizures and death from respiratory failure.

Tubers are the most toxic part of the plant, especially in early spring. The parsnip-like roots extending from the tuber are two to four times less toxic, and as the vegetative parts of the plant grow and mature, they become less toxic. Preliminary studies suggest that mature leaves and stems are much less toxic and after drying are non-toxic (Panter *et al.*, 1988). Historically, water hemlock was believed to be most dangerous in early spring, and poisoning usually occurred when animals milled around in streambeds or sloughs and exposed tubers, which were then ingested. Although this is true, a recent case of poisoning and death in cattle after ingesting flower and green seed heads implicates this phenological stage as dangerous also (Panter *et al.*, 2011). Chemical comparison of green seed and tubers and mouse bioassay studies showed that green seed was equally as toxic as tubers. Like tubers, the more mature vegetation, including leaves, flowers, and green seed heads, was very palatable. Free choice exposure of hamsters to the white parsnip-like roots suggests that they are quite palatable and are less toxic than the tuber. Observations of cattle grazing early in spring suggest that the young shoots of water hemlock are very palatable because young plants growing in streambeds were frequently and extensively grazed (Panter, personal observation).

Clinical signs of poisoning appear within 10–15 min after ingestion and progress from nervousness, frothing, ataxia, dyspnea, muscular tremors, and weakness to involuntary, spastic head and neck movements accompanied by rapid eye blinking and partial occlusion of the eyes from the nictitating membranes. This is quickly followed by collapse and intermittent grand mal seizures lasting 1 or 2 min each followed by relaxation periods of 8–10 min. Depending on the dosage, recovery may occur or seizures continue until death from exhaustion or respiratory failure. There appears to be a threshold response in which very small increases in dosage will induce an apparently normal animal into grand mal seizures (Panter *et al.*, 1996a).

Upon necropsy, gross lesions are confined to pale areas in heart muscle and skeletal muscles, particularly the long digital extensor muscle groups (Panter *et al.*, 1996a). Microscopic lesions include multifocal, subacute to chronic myocardial degeneration characterized by granular degeneration of myofiber cytoplasm necrosis and replacement fibrosis in the heart. These areas correspond to the pale areas observed grossly. There is bilateral symmetrical, subacute to chronic myofiber

degeneration and necrosis of the long digital extensor muscle groups. Clinical serum chemistry changes of elevated lactic dehydrogenase, aspartate aminotransferase, and creatine kinase occur in relation to severity of seizures. The extent of gross and microscopic lesions and clinical chemistries are a result of the severity of the seizures. Experimentally, barbiturates prevented seizures, death, and lesions in sheep, and a 3× lethal dose of water hemlock could be reversed with pentobarbital and no death occurred (Panter *et al.*, 1996a). In animals in which seizures were prevented by barbiturates, there were no lesions observed even though doses of two and three times the lethal dose were administered.

## Prevention and treatment

Prevention of poisoning is accomplished by recognizing the plant and avoiding exposing animals to it early in the spring or when in flower/seed stage. Water hemlock is easily controlled with herbicides (2,4-D per manufacturer's specification); however, herbicide use is often restricted near natural water sources. If few plants are present, hand pulling may be accomplished using caution to discard tubers away from possible exposure to animals or humans.

Successful treatment with barbiturates or perhaps tranquilizers prevents death and the lesions and serum chemistry changes; however, treatment must be prompt (Panter *et al.*, 1996a). This treatment has been successful in humans, but in animals it has never been demonstrated in the field and would require a veterinarian to be on sight soon after the ingestion of this plant.

## PONDEROSA PINE NEEDLES (PINUS SPP.)

The needles of ponderosa pine have been known for years to induce abortion in pregnant cows when grazed, particularly during the last trimester of pregnancy (Gardner *et al.*, 1999). Occasional toxicosis in pregnant cows occurs; however, cases of toxicosis in nonpregnant cows, steers, or bulls are not reported.

## Description

Ponderosa pine (*Pinus ponderosa*) is one of the most prevalent species of *Pinus* in the western United States. Under ideal conditions, it grows to heights of more than 60 m and 1.5–2.5 m in diameter. It is extensively harvested for lumber. During early growth, the bark is dark brown to

black, hence the name “black jack” pine. Older trees have a bark of cinnamon brown to yellow, hence the name “yellow” pine. Ponderosa pine is a three-needled pine, although groups of two and three can be found on the same tree. The needles are approximately 8–20 cm long growing in clusters. They are soft and quite palatable, especially in the winter, when green feed is limited. The cones are brown, 7–15 cm long, and frequently grow in clusters. Two varieties of ponderosa pine have been identified, var. *ponderosa* and var. *scopulorum*, along with a separate five-needled species *Pinus arizonica*.

## Distribution

Ponderosa pine grows in every state west of the Great Plains and western Canada. It has a total stand greater than any native tree in the western United States except Douglas fir. It grows at elevations between 1500 and 2500 m, although populations in California do extend almost to sea level. Ponderosa pine is drought resistant and is extensively harvested for lumber. Gardner and James (1999) surveyed numerous other *Pinus*, *Juniperus*, *Abies* species, etc. for isocupressic acid content, and Table 77.6 lists common name,

TABLE 77.6 Concentration of isocupressic acid (ICA) and related metabolic compounds from selected species and locations

Species	Common name	Location	ICA concentration (%DW)
<i>Abies concolor</i>	White fir	Arizona	n.d.
		California	n.d.
		Colorado	0.04
		Utah	n.d.
<i>Abies grandis</i>	Grand fir	Idaho	n.d.
		Oregon	n.d.
<i>Abies lasiocarpa</i>	Subalpine fir	Oregon	n.d.
		Colorado	n.d.
		Idaho	0.04
		Utah	n.d.
<i>Abies magnifica</i>	Red fir	California	0.05
<i>Cupressus macrocarpa</i>	Monterey cypress	California	n.d.–0.06
		New Zealand	0.89–1.24
<i>Cupressus X ovensii</i>	Ovens cypress	New Zealand	0.81
<i>Juniperus californica</i>	California juniper	California	0.93 needles
			0.05 bark
<i>Juniperus communis</i>	Mountain common juniper	Colorado	2.05–2.88
		Utah	1.50–5.0
<i>Juniperus monosperma</i>	One seed juniper	Arizona	0.14
		New Mexico	n.d.
<i>Juniperus occidentalis</i>	Western juniper	Oregon	0.10
		California	Imbricatoloic acid = 1.0
			0.10
<i>Juniperus osteosperma</i>	Utah juniper	Utah	Imbricatoloic acid = 1.0
			1.83 stems = total labdane acids
			n.d.
			0.07
			n.d.
<i>Juniperus scopulorum</i>	Rocky mountain juniper	Colorado	n.d.
		Utah	Agathic acid = 1.50
		Utah	0.84
		New Mexico	0.33
		Arizona	0.42
<i>Juniperus virginiana</i>	Eastern red cedar	Nebraska	Needles, low
			bark, <0.10 – high
<i>Larix occidentalis</i>	Western larch	Oregon	n.d.
<i>Libocedrus decurrens</i>	Incense cedar	Oregon	0.07
<i>Picea engelmannii</i>	Engelmann spruce	California	0.27
		Colorado	n.d.
		Idaho	0.04
		Montana	0.31
		Oregon	n.d.
		Utah	n.d.
		Utah	n.d.
<i>Picea pungens</i>	Colorado blue spruce	Utah	0.17
<i>Pinus aristata</i>	Bristle cone pine	Colorado	n.d.
		Colorado	0.01–0.05

(Continued)



TABLE 77.6 (Continued)

Species	Common name	Location	ICA concentration (%DW)
<i>Pinus arizonica</i>	Arizona pine	California	n.d.
		Arizona	n.d.
<i>Pinus contorta</i>	Lodgepole pine	Oregon	0.28
		Idaho	0.11
		Colorado	0.29–0.47
		Utah	0.66
		Canada (British Columbia)	0.45
<i>Pinus densiflora</i>	Japanese red pine	Korea	n.d.
<i>Pinus echinata</i>	Short leaf pine	Arkansas	n.d.
<i>Pinus edulis</i>	Pinyon pine	Arizona	n.d.
		Colorado	0.12
		New Mexico	0.10
		Utah	0.45
<i>Pinus elliottii</i>	Slash pine	Arkansas	n.d.
<i>Pinus flexilis</i>	Limber pine	Colorado	n.d.–0.06
		Utah	n.d.
<i>Pinus halepensis</i>	Aleppo pine	California	n.d.
<i>Pinus jeffreyi</i>	Jeffrey pine	California	0.04–0.54
<i>Pinus koraiensis</i>	Korean pine	Utah	Positive
		Korea	0.02
<i>Pinus monophylla</i>	Single-leaf pinyon	Nevada	0.32
<i>Pinus montezumae</i>	Montezuma pine	California	n.d.
<i>Pinus palustris</i>	Long-leaf pine	Arkansas	n.d.
<i>Pinus patula</i>	Patula pine	South Africa	<0.10
<i>Pinus ponderosa</i>	Ponderosa pine	Oregon	0.74–1.30
		Arizona	0.49
		California	0.08–1.35
		Utah	0.51
		Colorado	0.49–0.58
		South Dakota	0.10–1.30
		Wyoming	0.58–1.11
		Germany	0.62
<i>Pinus radiata</i>	Radiata pine	New Zealand	n.d.–0.26
<i>Pinus strobus</i>	White pine		n.d.
<i>Pinus taeda</i>	Loblolly pine	Arizona	n.d.
		Arkansas	n.d.
<i>Pseudotsuga menziesii</i>	Douglas fir	Utah	0.04
		Colorado	0.05
		California	n.d.
		Idaho	n.d.
		Arizona	n.d.
		Oregon	n.d.
<i>Thuja plicata</i>	Western red cedar	Arizona	0.42
		New Mexico	0.33
		Utah	0.84
		Germany	n.d.
<i>Tsuga mertensiana</i>	Mountain hemlock	Oregon	n.d.

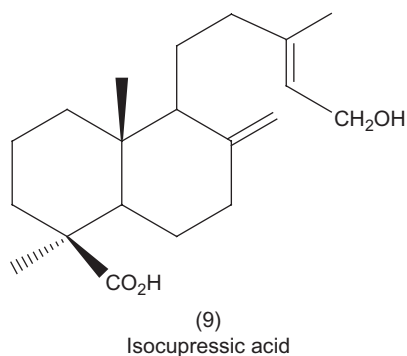
n.d., not detected (&lt;0.01%).

location, and isocupressic acid concentration of many species of trees or shrubs. The bark from both Utah juniper and western juniper trees has also been found to be abortifacient to cattle (Gardner *et al.*, 2010; Welch *et al.*, 2011a).

*Pinus ponderosa* var. *ponderosa* (western variety) extends from the mountains of southern California northward on the western and eastern sides of the Sierra

Nevada–Cascade crest to Canada. The eastern variety *P. ponderosa* var. *scopulorum* meets the western variety near the Continental Divide in west-central Montana and extends southward throughout mountains, plains, and basins to scattered stands in the Sierra Madre Occidental and Sierra Madre Oriental of northern Mexico. *Pinus arizonica* (once considered a third variety) has scattered populations in southern Arizona and New Mexico.

## Toxicology



The toxin in ponderosa pine that induces abortion in cattle is the labdane resin acid isocupressic acid (9) (ICA; Gardner *et al.*, 1994). Two related derivatives (succinyl ICA and acetyl ICA) also contribute to the induction of abortion after hydrolytic conversion to ICA in the rumen (Gardner *et al.*, 1996). Other related labdane acids (agathic acid, imbricatoloic acid, and dihydroagathic acid) that are found in ponderosa pine needles at low levels may also contain abortifacient properties based on their similar chemical structure to ICA. Other genera and species have also been implicated in abortions, such as Monterey cypress (Parton *et al.*, 1996), Korean pine (Kim *et al.*, 2003), and California juniper and lodgepole pine (Panter, personal communications). Table 77.6 summarizes current information on plant species analyzed for ICA and/or ICA derivatives believed to contribute to abortions. Current research indicates that the concentration of ICA in ponderosa pine needles is not uniform throughout the same tree, the concentration of ICA in ponderosa pine needles can vary from location to location, and there is evidence for seasonal fluctuations as well (Cook *et al.*, 2010).

The primary toxicological effects of ponderosa pine needles in cattle are abortion and complications associated with abortion, such as retained fetal membranes, metritis, and occasional overt toxicosis and death (Gardner *et al.*, 1999). The abortions generally occur in the last trimester of pregnancy in the late fall, winter, or early spring. Abortions have been induced as early as 3 months of gestation and have been reported by ranchers to occur any time; however, the closer to the time of normal parturition that ingestion of pine needles occurs, the higher the risk of abortion. Abortions may occur following a single exposure to the needles, but results from controlled experiments indicate the highest incidence of abortion is in cows eating the needles over a period of days. Abortions have been associated with grazing of green needles from trees, slash from the lumber industry, and dead, dry needles from the ground.

Abortions are generally characterized by weak uterine contractions, uterine bleeding, incomplete cervical

dilation, dystocia, birth of weak but viable calves, agalactia, and retained fetal membranes (Gardner *et al.*, 1999). Two syndromes seem to occur depending on the amount of pine needles eaten. In the first syndrome, abortion occurs relatively quickly (3–6 days) with no signs of pending abortion, such as udder filling, vulvar swelling, or pelvic relaxation, but a small weak calf is quickly delivered. In the second syndrome, abortion occurs after 6–14 days, and all the previously mentioned signs occur before the calf is born. Unless complications occur, most calves are born alive, and the closer to normal parturition, the higher the survival rate. Calves born after 255 days of gestation will often survive with extra care but need to be supplemented with colostrum and milk from other sources until the dam begins to lactate. Cows with retained fetal membranes may need antibiotic therapy to avoid uterine infections.

Pine needles will induce abortion in buffalo, but sheep, elk, and goats do not abort. Pine needles, pine bark, and new growth tips of branches are all abortifacient and new growth tips are also toxic (Panter *et al.*, 1990b).

A separate toxic syndrome has been described in addition to abortion in which the abietane-type diterpene resin acids cause depression, feed refusal, weakness, neurological problems, and, eventually, death. Specific compounds include abietic acid, dehydroabietic acid, and other related compounds (Stegelmeier *et al.*, 1996). At 15–30% of the diet, pine needles have been shown to alter rumen microflora and affect the rumen fermentation (Pfister *et al.*, 1992). Rumen stasis is part of the toxic syndrome (Stegelmeier *et al.*, 1996).

Pine needle-induced abortion appears to mimic normal parturition except premature. The mechanism of action appears to be a reduction in blood flow to the caruncular vascular bed stimulating the fetal parturition mechanism (Ford *et al.*, 1992). ICA has not been found to be directly vasoactive, and it is unclear if ICA metabolites are vasoactive or if the vasoconstriction is a secondary response to ICA or ICA metabolites. Further research is needed to determine the cellular or biochemical mechanism and whether metabolic alteration of the ICA occurs.

Extensive vasoconstriction of the caruncular vascular bed with accompanying necrosis and hemorrhage are the only reported pathological changes in maternal tissues. These findings are supported by Ford *et al.* (1992), who reported that serum from pregnant cows fed pine needles showed vaso (vasoconstriction) activity *in vitro*.

## Prevention and treatment

The only recommendation to prevent pine needle abortion is to avoid grazing pregnant cows around pine trees, especially in the third trimester. There is no known

treatment for cattle once ingestion of pine needles has occurred. Open cows, steers, or bulls are apparently unaffected by pine needles; likewise, sheep, goats (pregnant or not), and horses can graze pine needles with impunity and experience no adverse effects. Supportive therapy (antibiotic treatment or uterine infusion for retained fetal membranes) is recommended for cows that have aborted, and intensive care of the calf may save its life. Grazing of pine needles intensifies during cold inclement weather and if other forage is in short supply. In spring, before green grass is available, cows will leave feeding grounds in search of new green grass and frequently graze old, dry needles from surrounding trees where the snow has melted. These cows are at risk and should be kept away from the pines. Research has also determined that low body conditioned cows are more likely to eat pine needles than cattle in adequate body condition (Pfister *et al.*, 2008b). Consequently, it is recommended that pregnant cattle grazing in ponderosa pine areas be maintained in good body condition (Pfister *et al.*, 2008b). Anecdotal information suggests that pregnant llamas may be at risk from pine needles, but no experimental support has been presented (Panter, personal communications).

## BROOM SNAKEWEED (*GUTIERREZIA* SPP.)

Broom snakeweed causes significant losses to cattle, sheep, and goat producers in Texas and New Mexico from abortions and toxicoses. There are some similarities with ponderosa pine needles, except pine needles apparently affect only cattle.

### Description and distribution

There are two major species of broom snakeweed, *Gutierrezia sarothrae* (perennial snakeweed or turpentine weed) and *G. microcephala* (threadleaf broomweed). Snakeweeds are short-lived perennial half shrubs ranging from 15 to 60 cm tall (Ralphs and McDaniel, 2011). Many unbranched erect stems originate from a woody base and die back when the plant enters dormancy. They have a suffrutescent growth form, with new stems originating from the crown each year without becoming woody. The stems originate from the crown and are unbranched, giving rise to the common name of broom snakeweed, with the stems resembling the straight straws of a broom. Leaves are narrow and linear and alternately arranged on stems. These species are of the Composite family; thus, they have two types of flowers.

Disc flowers are tightly clustered in the center of the head, whereas long yellow petal ray flowers radiate out from the head (Lane, 1985).

### *Broom snakeweed (Gutierrezia sarothrae (Pursh) Britt. & Rusby)*

Broom snakeweed has more than three florets (usually seven) per involucre or flowering cluster. Broom snakeweed is one of the most ubiquitous range plants and widely distributed throughout North America, ranging from the cold temperate climate of Canada to subtropical areas of Mexico and from the subhumid Great Plains to the montane Rocky Mountains and Sierras and the arid Great Basin. It ranges in elevation from 50 to 2900 m, rainfall from 20 to 50 cm, and temperatures from 4 to 21°C. It is a principal component of the following plant communities: desert creosote, desert grassland, short-grass prairie, salt-desert shrub, sagebrush, pinyon-juniper, and mountain brush. Its wide tolerance limits allow it to inhabit a broad range of environments, and it can dominate and cause significant problems as both a noxious and a poisonous weed in the short-grass prairies and sagebrush plant communities.

### *Threadleaf snakeweed (G. microcephala (DC) Gray)*

Threadleaf snakeweed has one floret per flowering cluster. Threadleaf snakeweed is restricted to the Mojave, Sonoran, and Chihuahuan deserts of the southwestern United States and northern Mexico. It grows in dry, desert habitat and favors sandy soils, and it has a greater tolerance for arid climates than does broom snakeweed.

### Ecology

Broom snakeweed is a native range shrub found throughout semiarid rangelands of the western United States. Although a minor component of pristine plant communities, it can predominate on rocky ridges, gravely slopes, and immature infertile soils. Ralphs and McDaniel (2011) provide an up-to-date review of broom snakeweed ecology, including seed ecology, population cycles, toxicology, and management. Broom snakeweed increases and may dominate rangelands following disturbances such as overgrazing, fire, and drought (Ralphs and Banks, 2009). Broom snakeweed will increase where it is established and will invade deeper soils and more productive habitats. It is very competitive with desirable grasses and greatly suppresses forage production.

A two-tier root system allows the deep taproot to extract deep-stored water, and the extensive adventitious roots near the surface take advantage of the light sporadic rain showers. It has little leaf stomatal control; thus, it is a luxuriant water user for maximum growth.

It grows early in the spring and depletes moisture from the entire soil profile before warm-season grasses break dormancy. Broom snakeweed is one of the most undesirable plants in the various regions of the west. On many localized rangelands of the southwest, it is the most significant problem limiting forage and livestock production.

Broom snakeweed is short-lived and experiences dramatic population cycles, which appear to be related to climatic patterns. Although it is very competitive for soil moisture, it is not particularly drought tolerant. Broom snakeweed populations die off in drought and from insect depredation, but it is one of the first plants to germinate and establish when rains resume, forming widespread monospecific stands (Ralphs and Sanders, 2002).

## Toxicology

The snakeweeds are toxic and abortifacient to cattle, sheep, and goats. Abortions and retained fetal membranes in cattle are among the most serious problems in livestock. In 1985, McGinty estimated losses in excess of \$15 million annually to the cattle industry in Texas alone and more than \$30 million when losses in New Mexico and Arizona were included. This does not account for indirect losses, such as loss of usable forage, management changes, increased calving intervals, or added veterinary care.

Snakeweed contains toxic and abortifacient compounds. Extracts of a saponin fraction from threadleaf snakeweed caused abortions in rabbits, goats, and cattle at low doses and caused death at high doses. Molyneux *et al.* (1980) identified some major monoterpenes and sesquiterpenes in the essential oil fraction of snake-weed, including  $\alpha$ -pinene, myrcene, linalool, *cis*-verbenol, *trans*-verbenol, verbenone, geraniol, caryophyllene, and  $\gamma$ -humulene. Several furanoditerpene acids and flavones from the resinous exudate in trichomes on leaves of broom snakeweed have been identified. These diterpene acids were structurally similar to ICA, the abortifacient compound in ponderosa pine (*P. ponderosa* Laws) needles. Gardner *et al.* (1999) speculated that some of the furanoditerpene acids may be abortifacient, whereas others may be toxic, and the relative concentrations determine whether animals are poisoned or abort. The crude resin content of broom snakeweed, which includes the diterpene acids along with other monoterpenes, increased from 5% in early growth up to 13% at flowering in August (Ralphs *et al.*, 2007).

Snakeweeds are both abortifacient and toxic. In west Texas in the 1930s, 10–60% of cattle ranches experienced abortions, and cows retained placentas, which led to infection and death of the cows. Many calves were born small and weak. Threadleaf snakeweed was fed to cattle, sheep, and goats, causing damage to the liver and

kidneys, but no abortions were produced. Dollahite and Anthony (1957) and others found that threadleaf snakeweed did cause abortions, retained placenta, and weak calves, and it was more toxic on sandy soils. Low levels of snakeweed cause abortions and high levels are toxic. Clinical signs of poisoning include anorexia, mucopurulent nasal discharge, loss of appetite and listlessness, diarrhea, and then constipation and rumen stasis, which may lead to death.

There appears to be a relationship between nutrition and fertility problems caused by broom snakeweed. Smith *et al.* (1991) summarized research in rats at New Mexico State University and concluded that increasing amounts of snakeweed in rat diets reduced intake, which led to malnutrition and contributed to diminished fertility and increased fetal mortality. Edrington *et al.* (1993) confirmed that increasing amounts of snakeweed in rat diets reduced intake and contributed to problems of malnutrition and toxicity. However, they determined that the overriding factor in reducing fertility and reproduction was the impaired hormone balance and disruption of blood flow to the uterus and developing embryos. Ewes on a high-quality alfalfa diet (18% crude protein) consumed snakeweed for up to 25% of the ration with no adverse effects on estrus; whereas ewes fed blue grama hay (11% crude protein) would not consume rations containing more than 10% snakeweed, and 43% of these ewes did not show estrus and did not breed. In heifers fed snakeweed as 15% of a balanced diet before breeding and during early gestation, there was no effect on progesterone levels or conception rates. During the last trimester of gestation, snakeweed added up to 30% of this same diet did not cause abortion or lower calf birth weight. In a grazing trial on snakeweed-infested crested wheatgrass, cows in the last trimester of gestation were forced to graze snakeweed as a biological control. Snakeweed consumption averaged 10% of bites over the day and peaked at 20% of bites in the evening grazing periods. There were no signs of toxicity or abortions, even though their feed intake was severely restricted (Ralphs *et al.*, 2007).

## Management and treatment

Broom snakeweed is usually not palatable to most large ungulates; cattle will not graze snakeweed unless all other vegetation is depleted (Ralphs *et al.*, 2007). Thus, the management strategy to prevent grazing of broom snakeweed and prevent toxicosis is simple: ensure adequate feed is available. This can be accomplished by maintaining range in good condition and moving animals when proper utilization is reached. Although this sounds relatively simple, there are thousands of acres of rangeland on which broom snakeweed is dominant;



thus, how can these ranges be restored or utilized by livestock or wildlife?

Thacker *et al.* (2008) studied the invasion of broom snakeweed in two plant communities during a 5-year period following disturbance and evaluated vegetation change in a state-and-transition model. In a sagebrush/bunchgrass community that burned, bluebunch wheatgrass became the dominant species, and in the sagebrush-only dominated community that burned, broom snakeweed quickly established and dominated the community. Snakeweed cover increased from 2 to 31% during the 5-year period.

Broom snakeweed can be controlled by the common rangeland herbicides: Tordon (0.25–0.5lb/ac) is most consistent, Escort (3–6oz./ac) is a promising herbicide, and 2,4-D (1–2lb/ac) can be applied for two successive years when soil moisture is not limiting growth (Whitson and Freeburn, 1990). Better control is obtained in fall after flowering, when the carbohydrate stream is going down and carries the herbicide to the roots.

Ralphs and Banks (2009) used intense short-duration grazing pressure and high stocking rates to force cattle to graze snakeweed in an attempt to reduce snakeweed dominance. This intense targeted grazing pressure actually reduced snakeweed plants, but this method of snakeweed control has yet to be recommended, especially considering the health of the animals and the potential for reestablishment of snakeweed. Although the long-term success of targeted grazing to control snakeweed is yet to be determined, one should strongly consider seeding with cool-season grasses following episodes of overgrazing or targeted grazing such as reported by Ralphs and Banks. Defoliation studies to mimic grazing suggested that spring treatments put snakeweed at a disadvantage compared to fall clipping (Ralphs, 2009). Although there was little difference in the effect of clipping cool-season grasses in the spring versus the fall, there was a distinct effect on snakeweed.

Snakeweed is readily killed by fire, and prescribed burning is an effective control in the early stages of the population cycle while there is sufficient grass to carry a fire and respond to the released resources. In the latter part of the population cycle, grasses will be crowded out, and spraying with herbicides may be the only alternative. In this case, spraying must be followed by seeding to ensure a weed-resistant plant community is established that will resist reinvasion of snakeweed. Cool-season grasses such as crested, pubescent, and bluebunch wheatgrass will compete with snakeweed seedlings. Following drought, wildfires, or herbicide control, it was recommended that cool-season grasses should be seeded in an attempt to establish a solid stand that will prevent snakeweed establishment and subsequent domination in the plant community. If this approach is not successful, downy brome will likely fill

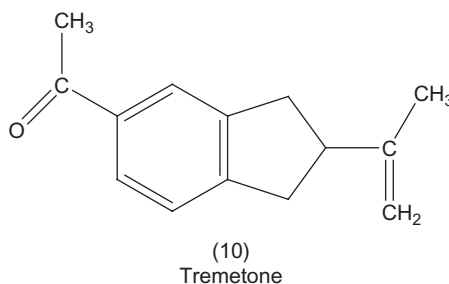
in, which will also often suppress the establishment of broom snakeweed.

Insects may play a role in snakeweed population die-off. Many insects are associated with snakeweed: defoliators (leaf tyers, grasshoppers, and weevils), sap suckers (scale insects and mealybugs), and root-boring weevils and gall formers. Individually and collectively, they contribute to the demise of mature snakeweed populations. However, it is unlikely that they will contribute to preventing a population buildup because of the time lag of their populations in relation to that of snakeweed.

Treatment of sick animals is only symptomatic, providing supplementation to weak calves and antibiotic therapy to cows with retained fetal membranes to avoid infection.

## RAYLESS GOLDENROD (*HAPLOPAPPUS HETEROPHYLLUS*)

Rayless goldenrod (*Haplopappus heterophyllus*) is a toxic range plant of the southwestern United States. The disease associated with toxicity has been referred to as “alkali disease” because originally it was associated with drinking of alkali water. Currently, it is referred to as “milk sickness” or “trembles” (the same as white snake-root in the Midwest) because the toxin tremetone (10) is excreted in the milk and subsequently results in poisoning of humans and nursing offspring. *Haplopappus acradenius* was implicated in poisoning in cattle in southern California (Galey *et al.*, 1991).



### Description

Rayless goldenrod is an erect, bushy, unbranched perennial shrub that grows 0.5–1.5m tall. The base is woody, and leaves alternate linearly up the stem. Heads are numerous, small, and clustered at the top of the stem, with 7–15 yellow flowers on each head.

### Distribution

Rayless goldenrod grows in the desert rangelands of the southwestern United States from southern Colorado into

Texas, New Mexico, Arizona, and California into Mexico. The plant grows abundantly on alkaline and gypsic soils in western Texas and the Pecos River Valley.

## Toxicology

The toxic constituents of rayless goldenrod are similar to those found in white snakeroot, and the original term, tremetol, is a mixture of ketones and alcohols. Tremetone (5-acetyl-2,3-dihydro-2-isopropenyl-benzofuran) was thought to be the principle toxic factor; however, 11 different compounds have now been isolated and identified (Lee *et al.*, 2010). This elucidation of different chemotypes of white snakeroot partially explains the sporadic and unpredictable toxicoses reported in livestock throughout the midwestern United States.

The toxicity of rayless goldenrod mimics that of white snakeroot. Clinical signs of poisoning may occur after ingestion of 1–15% BW during a 1- to 3-week period. Signs begin with depression or inactivity, followed by noticeable trembling of the fine muscles of the nose and legs. Most cases of poisoning reported constipation, nausea, vomiting, rapid labored respiration, progressive muscular weakness, stiff gait, standing in a humped-up position, dribbling urine, inability to stand, coma, and death. Signs are similar in cattle, sheep, and goats. The disease is often more acute and severe in horses than in cattle, and horses may die of heart failure after subacute ingestion of white snakeroot and presumably rayless goldenrod. Cattle have also been poisoned on a related plant (*Haplopappus acradenius*) in southern California (Galey *et al.*, 1991). In this case, 21 of 60 cattle died and 15 of 60 were affected but recovered. Creatine phosphokinase and ketones were elevated, and severe myonecrosis was described in the dead animals.

## Prevention and treatment

Rayless goldenrod is not readily palatable, and toxicity results from animals being forced to graze the plant due to lack of good quality forage. Avoiding overgrazing will usually minimize poisoning in livestock.

Control of rayless goldenrod can be accomplished with herbicide applications. Late summer and early fall application of picloram or 2,4-D ester is successful in reducing plant populations.

Treatment is generally symptomatic and supportive, providing dry bedding, good shelter, and fresh feed and water. Activated charcoal and saline cathartic may be beneficial. Treatment may include fluids, B vitamins, ketosis therapy, and tube feeding. Hay and water should be placed within reach if the animal is recumbent. In lactating cows, frequent milking may facilitate a more

rapid elimination of the toxins. In horses, monitoring of cardiac arrhythmias and electrocardiogram will provide information concerning heart damage and associated circulatory dysfunction. Treatment is the same for rayless goldenrod and white snakeroot because the toxins are the same.

## HALOGETON (*HALOGETON GLOMERATUS*)

Halogeton is an alien, invasive, noxious, and poisonous weed introduced from central Asia in the early 20th century. It was first collected along a railroad spur near Wells, Nevada, in 1934 and rapidly invaded 11.2 million acres of the cold deserts of the western United States (Young, 1999). There has been no appreciable spread since the 1980s because halogeton has filled all the suitable niches within its tolerance limits. It currently infests disturbed areas within the salt-desert shrub and sagebrush plant communities in the Great Basin, Colorado Plateau, and Wyoming's Red Desert physiographic provinces, which have 3–15 in. of annual precipitation.

Halogeton's infamy began in the 1940s and 1950s by causing large, catastrophic sheep losses. There were many instances of large dramatic losses; sometimes entire bands of sheep died overnight from halogeton poisoning. *Life* magazine ran a cover story titled "Stock Killing Weed" that focused national attention on halogeton. Congress passed the Halogeton Act in 1952 with the intent to

- detect the presence of halogeton;
- determine its effects on livestock; and
- control, suppress, and eradicate this stock-killing weed.

Federal research was reallocated from the Forest Service Experiment Stations to the Bureau of Plant Industries, creating the Range Research unit devoted specifically to "solving" the halogeton problem. It was realized that halogeton was not the problem but a symptom of a larger problem – that of degradation of desert rangelands (Young, 1999). It invaded disturbed sites where sheep congregated – around railroad loading sites, trail heads, stock drive ways, and water holes. When hungry sheep were turned loose to graze, halogeton was the only feed available, and they consumed too much, too rapidly, and were poisoned.

## Description and ecology

Halogeton is an annual plant germinating from seed each year. Its stems are branched from the base and

tinged with red and purple. Its leaves are fleshy and “hot dog” shaped, with the distinguishing feature of a single spine on its tip. It can be distinguished from Russian thistle and pigweed (with which it grows) by the tubular, spine-tipped leaf. It does not have flowers but, rather, bracteoles formed in the axils of leaves from which seed clusters develop. These seed clusters occur throughout the length of its stems. A robust plant may have 1500 linear inches of stem, with 75 seeds/in., producing 200–400lb seed/acre. The seeds are winged and are spread by wind and rodents.

Its seeds are dimorphic, which is key to its successful survival strategy. The majority of its seeds are black and readily germinate anytime; temperature and soil moisture are favorable. However, the black seeds are viable for less than 1 year. A small percentage of the seeds are hard and brown with low germination rates, but they survive for long periods in the soil and germinate when favorable conditions return. The dimorphic seed provided abundant seed for germination each year but also a reserve if drought killed its populations over successive years.

Although halogeton will germinate whenever conditions are favorable, it remains as a seedling until May, at which time lateral branches develop and ascend. It reaches its reproductive stage by mid-summer, or when soil moisture becomes limiting. When mature, the winged fruits are blown by wind or carried and deposited by rodents.

Halogeton is not competitive with perennial shrubs and grasses. It will not invade healthy desert plant communities. When a site is disturbed, halogeton will invade and establish, and its allelopathic properties provide an added advantage to its invasive nature. It takes up sodium and potassium from saline soils, forming the respective oxalates. These oxalates provide an important metabolic function to maintain high cell sap osmotic potential to allow the plant to take up saline water. Oxalates accumulate during the growing season, reaching peak concentration in the fall (20–36% of plant dry weight). Soluble oxalates leach out of the senescent foliage during the winter and accumulate on the soil surface, increasing its salinity. Thus, halogeton modifies its environment, making it more saline to meet its requirements, while exceeding the tolerance limits of associated species.

## Toxicology

The toxins are sodium and potassium oxalates, and plants are high in these oxalates in the fall and early winter when sheep enter the desert winter ranges. Poisoning occurs when sheep consume more oxalates than the body can detoxify (James, 1999). Rumen microbes can

detoxify the oxalates, and their populations can be induced to accommodate increasing levels of oxalates. Furthermore, calcium in the native plants or Ca supplements will rapidly combine with the oxalates in the rumen to form Ca oxalates that cannot be absorbed and are excreted in feces. If the Na oxalates are absorbed, they can be flushed out in urine. If they reach the bloodstream, they precipitate the Ca from the blood, creating Ca oxalate crystals, causing hypocalcemia resulting in shock and death. The Ca oxalate crystals physically damage the tubules of the kidney. The Na oxalates interfere with two key enzymes (succinic dehydrogenase and lactic dehydrogenase) in the Krebs cycle, disrupting energy metabolism. Combined, they cause rapid and acute death.

Clinical signs of poisoning include depression, anorexia, weakness, incoordination, recumbency, blood-tinged nasal discharge, coma, and rapid death. Gross pathologic changes include hemorrhage and edema of the rumen wall, hyperemia of the abomasal wall, and intestinal mucosa and ascites. Morphologic changes include hemorrhage and calcium oxalate crystal formation in the rumen wall and oxalate crystals with accompanying cellular damage in the renal tubules of the kidney.

## Treatment of poisoned animals

Animals can be drenched with water to flush oxalates out in the urine, or including dicalcium phosphate in the drench provides Ca that will combine with oxalates in the rumen and can be excreted. Intravenous injection of calcium gluconate can maintain blood Ca levels, but the forming Ca oxalate crystals will continue to damage kidneys (James, 1999). However, it is generally impractical to treat a severely poisoned animal. Prevention is the key to avoid poisoning. Only hungry sheep are poisoned. Research has demonstrated that as little as 1 oz. of soluble oxalates can be lethal to fasted, hungry sheep. Well-fed sheep grazing nutritious forage throughout the day can tolerate more than 4 oz. of soluble oxalate. Sheep grazed in a desert plant community infested with halogeton consumed it from 5 to 25% of their diets without ill effect. If other forage is available, they will likely not get a lethal dose.

## Management to prevent poisoning

Never turn hungry sheep onto dense halogeton-infested sites. Provide good feed following trucking or trailing. Ensure there is good feed available following watering. Introduce sheep gradually to halogeton to allow rumen microbes to adjust. Some sheep producers graze their

sheep on shadscale ranges (which contain low oxalate levels) before going into halogeton areas. Do not overgraze; maintain desert range in good condition. This prevents halogeton invasion as well as provides an alternative food source.

Herbicide control is not recommended because the waxy surface of halogeton's leaves hinders absorption of most herbicides. More important, however, desirable desert shrubs are killed, leaving the site open for further invasion and degradation by halogeton and other invasive weeds.

## OAK POISONING

Toxicoses in cattle from ingestion of oak buds, leaves, and acorns occurs in many areas of the United States and Europe. Poisoning is usually seasonal, with ingestion of buds and leaves in spring and acorns in fall.

All oaks should be considered potentially toxic (Table 77.7). Oaks are perennial trees or woody shrubs. They grow in all areas of the United States, especially in the southwestern states of Texas, Arizona, and Utah, and they also grow in Europe and New Zealand.

### Conditions of poisoning

The most dangerous period for oak poisoning is during March and April when new foliage is sprouting. Poisoning of cattle and, less frequently, sheep and goats occurs on ranges of the southwest and especially during drought years when the forage is limited. Supplemental feeding so that oak ingestion is below 50% of their diet will usually prevent poisoning. Acorn poisoning occurs in the fall, particularly when an acorn crop is heavy. Cattle, sheep, horses, and swine have been involved, and craving for acorns has been observed in which animals appear to seek them out.

Swine seem to be somewhat resistant, and goats apparently utilize oak browse and are used to control oakbrush. Feeding high levels of immature gambel oak to goats did not induce any toxicosis.

The toxins in oak are tannins, particularly tannic acid and the phenolic acid gallic acid, and these are highest in new spring growth. Clinical signs of poisoning begin with depression, nasal discharge, abdominal pain, constipation, thirst, frequent urination, and rapid pulse, progressing over 1–3 days to rumen atony, anorexia, emaciation, weakness, prostration, rough coat, dry muzzle, subnormal temperature, bloody diarrhea, and death. Diagnosis is based on history of ingestion; clinical signs; elevated serum glutamic oxaloacetic transaminase, blood urea nitrogen, creatinine, and serum potassium; and lower urine specific gravity. Death may occur within 24 h of the onset of clinical signs.

Gross and microscopic lesions include gastritis, nephritis, increased peritoneal and pleural fluids, perirenal edema, and hemorrhages with pale swollen kidneys. The proximal convoluted tubules of the kidneys may be damaged, and abundant hyaline or granular casts may be evident. Mucous and blood are common in voided feces.

### Prevention and treatment

The best method of prevention is to provide adequate feed to reduce oak ingestion to less than 50% of the diet. Supplemental high-energy feed at 0.5–1 kg/head/day of a 54% cottonseed or soybean meal with 30% alfalfa meal, 6% vegetable oil, and 10% calcium hydroxide may be beneficial. Treatment includes blood transfusions if anemic, fluid therapy, rumenotomy if warranted, activated charcoal, plenty of fresh water, and avoiding stressing animals as much as possible.

## PYRROLIZIDINE ALKALOID-CONTAINING PLANTS

TABLE 77.7 Oak species believed to contribute to poisoning

Species	Common name
<i>Quercus gambelii</i>	Gambel's oak
<i>Q. harvardii</i>	Shin or shinnery oak
<i>Q. marilandica</i>	Jack oak
<i>Q. stellata</i>	Post oak
<i>Q. breviloba</i> or <i>durandii</i>	
<i>Q. coccinea</i>	Scarlet oak
<i>Q. pedunculata</i> or <i>robur</i>	European oak
<i>Q. prinus</i>	Chestnut oak
<i>Q. xubra</i>	Northern red oak
<i>Q. velutina</i>	Yellow-barked oak

Pyrrolizidine alkaloid (PA)-containing plants are numerous and worldwide in distribution and in toxic significance (Chee and Shull, 1985). Three plant families predominate in PA-producing genera and species: Compositae (*Senecio* spp.), Leguminosae (*Crotalaria* spp.), and Boraginaceae (*Heliotropium*, *Cynoglossum*, *Amsinckia*, *Echium*, and *Symphytum* spp.). All of these do not occur in the western United States. For plant descriptions, readers are referred to those species that cause losses in the western United States, which are listed in Table 77.8 with common names and distribution.



TABLE 77.8 PA-containing plants in the western United States: common names, habitat, and distribution

Scientific name	Common name	Habitat	Distribution
<i>Amsinckia intermedia</i>	Tarweed, fiddleneck	Dry, open cultivated fields	Pacific Coast; CA, WA, OR, ID
<i>Cynoglossum officinale</i>	Houndstongue	Dry, open areas; cultivated fields	Throughout West
<i>Echium vulgare</i>	Vipers bugloss		CA
<i>Symphytum officinale</i>	Comfrey		
<i>Senecio brasiliensis</i>			
<i>Senecio cineraria</i>	Dusty miller		Ornamental
<i>Senecio glabellus</i>	Bitterweed	Wet soils	TX and east
<i>Senecio integerrimus</i>			MT; Rocky Mountains, CA,
<i>Senecio jacobaea</i>	Stinking willy, tansy ragwort	Open fields and woods	Pacific Northwest
<i>Senecio longilobus</i>	Woody or groundleaf groundsel	Deserts	WY to NE, south to AZ and TX
<i>Senecio spartioides</i>	Broom groundsel		CO, UT south to TX, AZ, NM
<i>Senecio riddellii</i>	Riddell groundsel	Deserts	CO, UT south to TX, AZ, NM
<i>Senecio vulgaris</i>	Common groundsel		CA, OR
<i>Crotalaria sagittalis</i>			Pacific Northwest

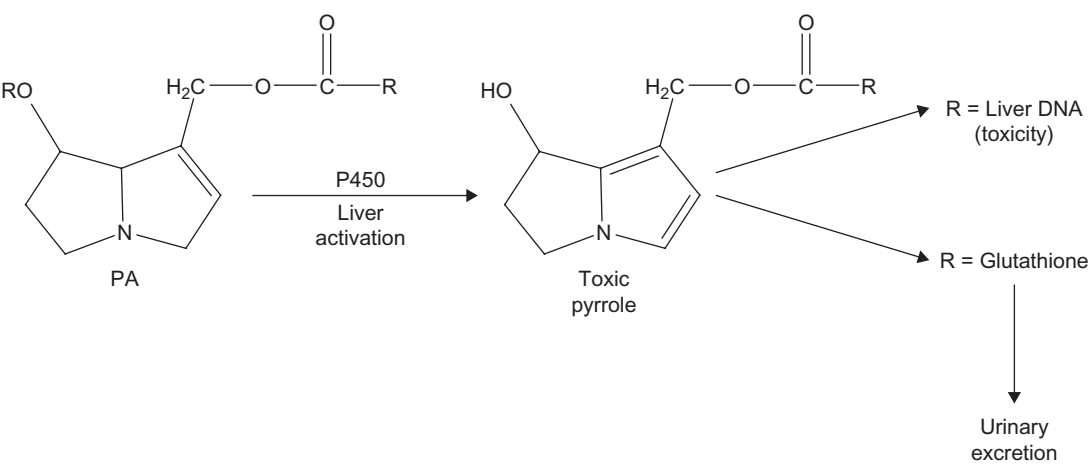


FIGURE 77.7 Metabolic pathway of pyrrolizidine alkaloids showing the toxic pyrrole pathway and glutathione conjugate pathway for excretion.

Toxicology

More than 150 PAs have been identified and structural characteristics elucidated. The PAs contain the pyrrolizidine nucleus and can be represented by the basic structures of senecionine and heliotrine. The toxic effects of all PAs are somewhat similar, although their potency varies due to their bioactivation in the liver to toxic metabolites called pyrroles (Figure 77.7). These pyrroles are powerful alkylating agents that react with cellular proteins and cross-link DNA, resulting in cellular dysfunction, abnormal mitosis, and tissue necrosis. The primary effect is hepatic damage; however, many alkaloid and species-specific extrahepatic lesions have been described. Small amounts of pyrrole may enter the blood and be transported to other tissues, but there is debate on this issue because most pyrroles are super-reactive and not likely to make it into the circulation

(Stegelmeier *et al.*, 1999). When PA metabolites circulate, they are probably protein adducts that may be recycled. Some alkaloids (monocrotaline) may come off their carrier blood proteins and damage other tissues such as lung. Pigs seem more prone to develop extrahepatic lesions.

Toxicity of *Senecio*, *Heliotropium*, and *Echium* is largely confined to the liver, whereas *Crotalaria* will also cause significant lung damage. Typical histologic lesions are swelling of hepatocytes, hepatocyte necrosis, periportal necrosis, megalocytosis (enlarged parenchymal cells), karyomegaly (enlarged nuclei) fibrosis, bile duct proliferation, and vascular fibrosis and occlusion. Hepatic cells may be 10–30 times normal size, and DNA content may be 200 times normal.

In most species affected by PA poisoning, the liver becomes hard, fibrotic, and smaller. Because of decreased bile secretion, bilirubin levels in the blood rise, causing

jaundice. Common clinical signs include ill thrift, depression, diarrhea, prolapsed rectum, ascites, edema in the GI tract, photosensitization, and aberrant behavior. In horses, "head pressing" or walking in straight lines regardless of obstacles in the path may occur. These neurological signs in horses are due to elevated blood ammonia from reduced liver function. PA poisoning may cause elevated blood ammonia, resulting in spongy degeneration of the central nervous system.

Elevated levels of serum enzymes such as alanine aminotransferase, aspartate aminotransferase,  $\gamma$ -glutamyl transferase, and alkaline phosphatase are reported (Stegelmeier *et al.*, 1999). Use of these tests for diagnosis is supportive but should not be relied on exclusively because they vary with animal species and other conditions. They may also be in the normal range even though liver damage has occurred, and they tend to be transient. Liver function tests such as bilirubin, bile acids, or sulfo-bromophthalein (BSP) clearance may be useful estimates of the extent of liver damage.

There are marked differences in susceptibility of livestock and laboratory animals to PA toxicosis. Cows are most sensitive, followed by horses, goats, and sheep, respectively. In small laboratory animals, rats are most sensitive, followed by rabbits, hamsters, guinea pigs, and gerbils, respectively. Among avian species, chickens and turkeys are highly susceptible, whereas Japanese quail are resistant (Chee and Shull, 1985).

Detoxification mechanisms of PAs generally involve the liver and GI tract. Evidence of ruminal detoxification in sheep suggests this contributes to the reduced toxicity in that species. There are also substantial species-specific differences in the rate of PA metabolism. Both probably contribute to species susceptibility. For example, *Echium* and *Heliotropium* PAs are easily degraded by certain rumen microflora, but there is little evidence of ruminal degradation of *Senecio* PAs. The PAs in *Senecio* are macrocyclic closed esters of retronecine as opposed to the open esters found in heliotridine. Therefore, the reason for the difference in *Senecio* toxicity between sheep and cattle is unlikely to be the rumen detoxification but more likely differences in species-specific enzymatic activation of *Senecio* PAs. For example, in *in vitro* studies, retrorsine metabolism has been shown to be high in those species that are most susceptible and lowest in animals of least susceptibility. In addition, in *in vivo* studies, it was demonstrated that a higher pyrrole production rate occurred in cattle compared to sheep. Simple induction of liver microsomal enzymes by phenobarbitone increased pyrrole production and increased PA toxicity (LD<sub>50</sub> in guinea pigs from >800 to 216 mg/kg). PA toxicity may disrupt other hepatic functions. Abnormal copper metabolism, coagulation, NH<sub>3</sub> metabolism, protein metabolism, etc. may be affected in PA poisoning.

## Prevention and treatment

Because there are no proven effective methods of prevention or treatment, avoidance of the plant and controlling plant populations with herbicides or biological control is essential. Resistance to PA toxicosis in some species suggests that the possibility may exist to increase resistance to PAs. Dietary factors such as increased protein, particularly those high in sulfur amino acids, had minor protective effects in some species. Antioxidants such as BHT and ethoxyquin induced increased detoxifying enzymes such as glutathione S-transferase and epoxide hydrolase. Zinc salts have been shown to provide some protection against hepatotoxicosis from sporidesmin or lupinosis in New Zealand and Australia, and zinc supplementation reduced toxicity in rats from *Senecio* alkaloids (Burrows and Tyrl, 2001; Knight and Walter, 2001).

Many of these plants were introduced either inadvertently or intentionally. Without natural predators to keep populations in check, they experienced explosive growth and distribution followed by epidemic proportions of toxicity. Introduction of biological controls and natural population controls have reduced many of the plant populations and thus toxicoses have declined. Sheep, a resistant species, have been used to graze plants, particularly *S. jacobaea*.

## PHOTOSENSITIZING PLANTS

Numerous plants cause photosensitization resulting in losses to the livestock industry. Photosensitization is the development of abnormally high reactivity to ultraviolet radiation or natural sunlight in the skin or mucous membranes. Primarily induced in livestock by various poisonous plants, the syndrome in livestock has been defined as primary and secondary photosensitization.

## Description and distribution

Photosensitizing plants are too numerous to describe individually, and readers are referred to taxonomic texts for plant description. Photosensitizing plants occur throughout the world and are common in the diets of livestock and people. Their distribution in the western United States, common names, and toxins are listed in Table 77.9.

## Toxicology

### Primary

In primary photosensitization, the photoreactive agent is absorbed directly from the plant and reaches the

TABLE 77.9    Photosensitizing plants of the western United States, listed as primary and secondary photosensitizers

Scientific name	Common name	Distribution	Toxin
<b>Primary photosensitizers</b>			
<i>Hypericum perforatum</i>	St. John's wort, Klamath weed	Pacific Coast states	Hypericin
<i>Fagopyrum sagittatum</i>	Buckwheat	Northwestern U.S.	Fagopyrin
<i>Cymopterus watsoni</i>	Spring parsley	Southwestern U.S.	Furocoumarins
<i>Ammi majus</i>	Bishop's weed	Southwestern U.S.	Furocoumarins
<b>Secondary photosensitizers</b>			
<i>Artemisia</i>	Sagebrush	Western U.S.	
<i>Tetradymia glabrata</i>	Spineless horsebrush	Western U.S.	
<i>Tetradymia canescens</i>	Gray horsebrush	Western U.S.	
<i>Agave lecheguilla</i>	Lechuguilla	Southwest	Saponins
<i>Nolina texana</i>	Sacahuiste	Southwest	Saponins
<i>Tribulus terrestris</i>	Puncture vine	Southwest	Saponins
<i>Trifolium hybridum</i>	Alsike clover	North, Midwest	
<i>Lantana</i> spp.	Lantana	Southwest	Saponins
<i>Panicum</i> spp.	Panic grass, Kleingrass	Western U.S.	
<i>Brassica napus</i>	Rape	Western U.S.	
<i>Senecio</i> spp.	Senecios	Western U.S.	PAs
<i>Cynoglossum officinale</i>	Houndstongue	Western U.S.	PAs
<i>Cooperia pedunculata</i>	Amaryllis family	Southwest U.S.	
<i>Thamnosma texana</i>	Dutchmans breeches	Southwest U.S.	Psoralens
<i>Kochia scoparia</i>	Kochia, burning bush		Saponins
<i>Descurania pinnata</i>	Tansymustard		Unknown

PAs, pyrrolizidine alkaloids.

peripheral circulation and skin, where it reacts with the ultraviolet rays of the sun and results in sunburn, particularly of unprotected areas of the body. Hypericin and fagopyrin are polyphenolic derivatives from St. John's wort and buckwheat, respectively, and are primary photodynamic agents (Cheeke and Shull, 1985). By definition, primary photosensitization does not induce hepatic damage. Most agents are ingested, but some may induce lesions through skin contact. Several of these plants are weedy in nature and can contaminate pastures and feed. Exposure to some plants is increasing as they are becoming widely used as herbal remedies and holistic medicines. In most cases, the photodynamic agent is absorbed from the digestive tract unchanged and reaches the skin in its "native" form (Stegelmeier, 2002).

There are drugs and other toxins known to cause primary photosensitization, and these should be considered in the differential diagnosis. Phenothiazine-induced photosensitization is most common in ruminants because the photodynamic agent is phenothiazine sulfoxide, a rumen metabolite. Clinical signs in addition to photosensitivity include corneal edema and kerato conjunctivitis from the phenothiazine sulfoxide excreted in tears and the aqueous humor. Other toxins associated with primary photosensitivity include thiazides, acriflavins, sulfonamides, tetracyclines, methylene blue, coal-tar derivatives, furosemide, promazine, chlorpromazine, quinindine, and some antimicrobial soaps (Stegelmeier, 2002).

Secondary

In secondary or hepatogenous photosensitization, the photoreactive agent is phylloerythrin, a degradation product of chlorophyll. Phylloerythrin is produced in the stomach of animals, especially ruminants, and absorbed into the bloodstream. In normal animals, the hepatocytes conjugate phylloerythrin and excrete it in the bile. However, if the liver is damaged or bile secretion is impaired, phylloerythrin accumulates in the liver, the blood, and subsequently the skin, causing photosensitivity. This is the most common cause of photosensitization in livestock and horses (Knight and Walter, 2001). Because chlorophyll is almost always present in the diet of livestock, the etiologic agent of secondary photosensitization is the hepatotoxic agent.

The dermatologic signs of photosensitization in livestock are similar regardless of the plant or toxicant involved. Degree or severity varies, depending on the amount of toxin or reactive phylloerythrin in the skin, degree of exposure to sunlight, and amount of normal physical photoprotection (hair and pigmentation). First signs in most animals are restlessness or discomfort from irritated skin, followed by photophobia, squinting, tearing, erythema, itching, and sloughing of skin in exposed areas (i.e., lips, ears, eyelids, udder, external genitalia, or white pigmented areas) (Burrows and Tyrl, 2001). Swelling in the head and ears (edema) of sheep after ingestion of *Tetradymia* has been referred to as big head.

It was determined that sheep grazing black sagebrush (*Artemisia nova*) before *Tetradymia* were three times more likely to develop this photosensitization. Tissue sloughing and serum leakage may occur where tissue damage is extensive. Primary photosensitization rarely results in death. However, in secondary or hepatogenic photosensitization, the severity of liver damage and secondary metabolic and neurologic changes of hepatic failure may ultimately result in death. Recovery may leave sunburned animals debilitated from scar tissue formation and wool or hair loss.

### Prevention and treatment

Prevention of poisoning lies in controlling plants with photosensitizing potential and providing adequate quality forage to animals. Treatment after poisoning involves removing animals from sun exposure, treating areas of necrosis and sunburn, antibiotic therapy, and supplementing young animals when access to sunburned udders is prevented because of nursing discomfort to dams. Identifying chronic hepatic disease is complicated because many of the serum markers for hepatic disease have returned to normal. As normal hepatocytes become replaced with fibrous connective tissue, there are fewer damaged cells to elevate serum enzymes. Percutaneous liver biopsies are invaluable in identifying and diagnosing these cases (Stegelmeier *et al.*, 1999).

Plant-induced hepatopathy generally results in characteristic histologic lesions. For example, pyrrolizidine alkaloids generally cause bridging portal fibrosis with hepatocellular necrosis, biliary proliferation, and megalocytosis. *Panicum* and *Tribulus* species generally produce a crystalline cholangiohepatitis. Liver biopsy also provides prognostic information. The degree of damage is correlated directly with the animal's ability to compensate, recover, and provide useful production. Note that the liver reacts to insult in a limited number of ways, and most histologic changes are not pathognomonic. Hepatic cirrhosis (necrosis, fibrosis, and biliary proliferation) involves nonspecific changes that can be initiated by a variety of toxic and infectious agents (Stegelmeier *et al.*, 1999).

## DEATH CAMAS

All death camas species are assumed to be toxic; however, variation in toxicity exists between species and even within species depending on season, climate, soils, and geographical locations. Poisoning in sheep, cattle, horses, pigs, fowl, and humans has been reported.



**FIGURE 77.8** Foothill death camas with leaf structure, flower head, and bulb.

The largest losses generally occur in sheep. Sheep are primarily affected because of their tendency to select forbs, particularly in early spring when they are turned onto range before grasses have emerged.

Death camas is generally not palatable to livestock but is one of the earliest species to emerge in the spring. Poisoning most frequently occurs in spring when other more palatable forage is not available, or on overgrazed ranges where there is a lack of more desirable forage. Poisonings have resulted due to management errors in which hungry animals were placed in death camas-infested areas (Panter *et al.*, 1987).

### Description, habitat, and geographical distribution

Foothill death camas is typical of the 15–20 species of *Zigadenus* in North America and Asia (Figure 77.8). A list of death camas species and their habitats, distributions, and growth periods is provided in Table 77.10. It is difficult to distinguish between species because they are taxonomically similar. A member of the lily family, death camas is a perennial, glabrous herb with basal V-shaped grass-like leaves growing from an onion-like bulb with a dark-colored outer coat. Stems produced at flowering are single, unbranched, sparingly leafed, and terminated by a terminal raceme of greenish-white, cream-colored, or pink inflorescence. The perianth is six-membered, consisting of three lanceolate or ovate sepals and three petals separate or united below, with one or two glands just above the base; six stamens; and three styles. Floral parts are persistent but winter as the fruits develop. The seed



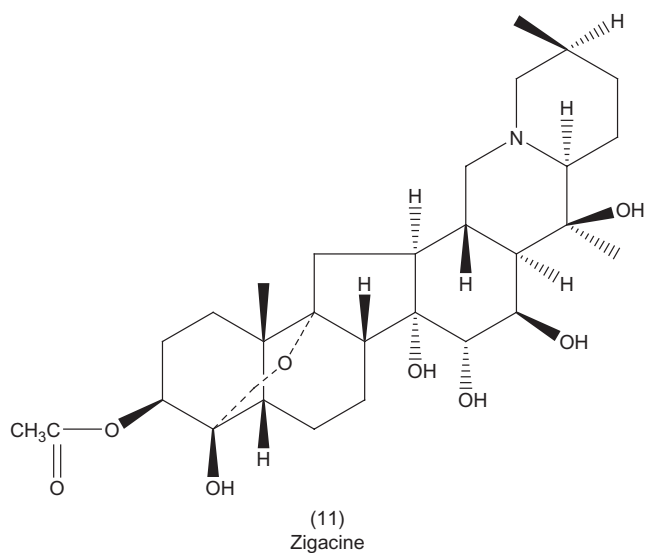
TABLE 77.10 *Zigadenus* spp.: common name, distribution, habitat, and growth periods

Species	Common name	Distribution	Habitat	Growth period
<i>Z. paniculatus</i> S. Watson	Foothill death camas; sand-corn; panicked death camas	Eastern WA, OR, ID, UT, WY, NV, eastern CA, northern AZ and NM	Foothills and benches	May–July
<i>Z. venenosus</i> S. Watson = <i>Z. gramineus</i> Rydb. = <i>Z. intermedium</i> Rydb. = <i>Z. salinus</i> Rydb.	Meadow death camas; grassy death camas	WA, OR, ID, MT, ND; south to NE, UT, CO, NV, CA; north to western Canada	Plains, prairies, meadows and open coniferous woods	May–July
<i>Z. nuttallii</i> A. Gray	Nuttall's death camas; poison camas	TN, AR, OK, KS, and northern TX	Prairies and rocky sites	April–June
<i>Z. gramineus</i> Rydb.	Grassy death camas	Southwestern Canada, MT, ID, WA; south through WY, CO, UT, NV, AZ, NM	Open hills and plains	April–July
<i>Z. elegans</i> Pursh = <i>Z. glaucus</i> Nutt.	White camas, elegant death camas, mountain death camas	AK, western Canada, MT, south into AZ and east into MN and IA	Prairies and meadows	June–August
<i>Z. leimanthoides</i> A. Grey	–	Southwestern U.S. and coastal plains of DE, NJ, and RI	Sandy pine lands and bogs of the coastal plains	June–August
<i>Z. fremontii</i> Torr.	–	Southwestern OR and western CA	Dry grassy or brushy slopes	May–July
<i>Z. glaberrimus</i> Michx.	–	Southeastern U.S.	Savannas and wet pine lands	July–Sept.
<i>Z. densus</i> Desr.	Black snakeroot, crow poison, St. Agnes' feather, black death camas	Southeastern U.S.	Damp soils, pine woods and bogs	May–June
<i>Z. exaltatus</i> Eastw. <i>Z. micranthus</i> Eastw.	– –	Central CA Northwestern CA, southwestern OR		May–June
<i>Z. vaginatus</i> Rydb.	Alcove death camas	Northwestern CA, southwestern OR		
<i>Z. virescens</i> Kunth.		AZ, NM, and northern Mexico		

is a three-cavited capsule, separating into three members and opening inwardly at maturity. Death camas is easily confused with wild onion, mariposa lily, or common camas particularly before flowering. Wild onions are distinguished by tubular leaves and their onion-like odor. The leaves of mariposa lilies are more U-shaped in cross section, and common camas has a blue flower. Death camas is prevalent in western North America and is native to the open plains and foothills of the United States.

### Toxicity of death camas to livestock

The toxins in death camas are of the cevanine steroidal alkaloid type – that is, zigacine (**11**). Zigacine is a very potent compound with an i.v. LD<sub>50</sub> of 2mg/kg and an oral LD<sub>50</sub> of 130mg/kg in mice (Welch *et al.*, 2011b). Clinical signs of toxicosis are similar in all livestock poisoned by *Zigadenus*, irrespective of the species of plant



involved. Excessive salivation is noted first, with foamy froth around the nose and muzzle that persists, followed by nausea and occasionally vomition in ruminants (Panter *et al.*, 1987). Intestinal peristalsis is dramatically increased, accompanied by frequent defecation and urination. Muscular weakness with accompanying ataxia, muscular fasciculations, prostration, and eventual death may follow. The pulse becomes rapid and weak, and the respiration rate increases but the amplitude is reduced. Some animals become cyanotic, and the spasmodic struggling for breath may be confused with convulsions. The heart fails before respiration, and at necropsy the heart is usually found in diastole. A comatose period may range from a few hours to a few days before death.

Pathological lesions are those of pulmonary congestion. Gross lesions of sheep include severe pulmonary congestion, hemorrhage, edema, and subcutaneous hemorrhage in the thoracic regions. Microscopic lesions include severe pulmonary congestion with infiltration of red blood cells in the alveolar spaces and edema. Diagnosis of poisoning may be established by clinical signs of toxicosis, evidence of death camas being grazed, histopathological analysis of tissues from necropsied animals, and identification of death camas in the rumen or stomach contents (Panter *et al.*, 1987).

Similarity in clinical signs of toxicosis between certain species of these plants suggests that the same alkaloids are present; however, differences in concentrations can explain the differences in relative toxicity of different species (Table 77.11).

## Management and prevention

Conditions conducive to poisoning by death camas include driving animals through death camas-infested ranges; not allowing animals to graze selectively; unloading hungry animals in infested areas; lambing, bedding, watering, or salting livestock in death camas-infested areas; or placing animals on range where little forage is available. Poisoning generally occurs in the early spring when death camas is the first green forage available and the young immature foliage is the most toxic. Single losses of 300–500 sheep have been reported (Panter *et al.*, 1987). In the 1987 case, 80% of the dead sheep were 80- to 90-lb lambs. Three key factors contributed to the losses: (1) ewes with lambs were driven through a heavily infested area of death camas when the sheep were hungry; (2) the sheep were bedded down for the night near the death camas area, providing immediate access to death camas the following morning; and (3) the herder panicked and rapidly forced the sheep out of the area, contributing to the stress and probably exacerbating the toxic effects and increasing the losses.

TABLE 77.11 Relative toxicity in sheep of five *Zigadenus* spp.

<i>Zigadenus</i> species	Average minimum toxic dose <sup>a</sup>	Average minimum lethal dose <sup>a</sup>
<i>Z. gramineus</i>	4	6
<i>Z. paniculatus</i>	10	25
<i>Z. venenosus</i>	4	20
<i>Z. elegans</i>	20	60
<i>Z. nuttallii</i>	2	5

<sup>a</sup>Grams of green plant per kilogram body weight.

## VERATRUM SPP.

*Veratrum* belongs to the Liliaceae (Lily) family and is composed of at least five species in North America. During the mid-20th century, up to 25% of pregnant ewes that grazed on pastures infested with *Veratrum californicum* in the mountains of central Idaho gave birth to lambs with serious craniofacial malformations. These malformations ranged from the gross anomaly of cyclops to less severe deformities of the upper and lower jaws. The Basque shepherds called the cyclopic defect “chatto,” which translated as “monkey faced” lamb disease. Although losses from *Veratrum* have long been reduced or eliminated on these ranges due to the application of research findings and recommended management strategies, biomedical research using the alkaloids, isolated and identified at the Poisonous Plant Research Laboratory, as molecular probes has opened a new frontier for human medical research (James *et al.*, 2004).

## Distribution

*Veratrum californicum* grows primarily in the high mountain ranges of the western United States (Knight and Walter, 2001). *Veratrum viride* is the most widespread species and grows in the northwestern United States north through western Canada into Alaska and is also widespread in the northeastern United States. *Veratrum insolitum* grows in a relatively small region of northwestern California and southwestern Oregon; *V. parviflorum* grows in the central southeastern states; and *V. woodii* grows from Ohio to Missouri, Oklahoma, and Arkansas. Two other species have been reported to cause poisoning in other countries: *V. japonicum* in Korea and *V. album* in Europe. Common names include western false hellebore, hellebore, skunk cabbage, corn lily, Indian poke, and wolfsbane. Caution should be used with common names because they may be used interchangeably within this genera but also in unrelated genera. For example, the name hellebore is also used for the genus *Helleborus* in the buttercup family.

## Habitat and description

Most *Veratrum* spp. are found in similar habitats of moist, open alpine meadows or open woodlands, marshes, along waterways, in swamps or bogs, and along lake edges in high mountain ranges. Most species grow at higher elevations. All species are similar, with coarse, erect plants approximately 1–2.5 m tall with short perennial rootstalks. The leaves are smooth, alternate, parallel veined, broadly oval to lanceolate, up to 30 cm long, 15 cm wide, in three ranks, and sheathed at the base. The inflorescence is panicle flowers; the lower ones often staminate and the upper ones perfect. The flowers of *V. viride* are distinctly green, and the fruit is three-chambered with several seeds.

## Toxicology

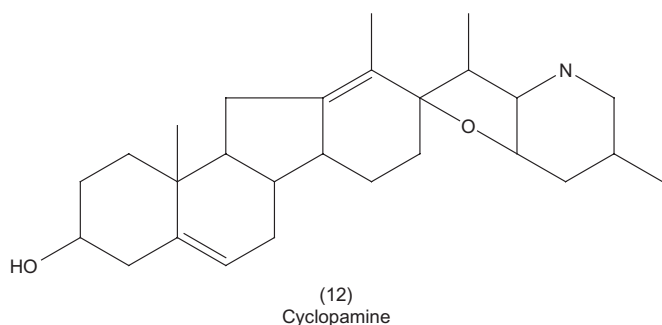
More than 50 complex steroidal alkaloids have been identified from the *Veratrum* spp. Five classes of steroidal alkaloids have been characterized: veratrines, cevanines, jervanines, solanidines, and cholestanes. The veratrines and cevanines are of considerable interest in toxicology because they are neurological toxins and hypotensive agents that bind to sodium channels, delaying closure and causing cardiotoxic and respiratory effects. The cevanine alkaloids, such as zigacine, are also found in *Zigadenus* spp., which are also members of the Lily family. The jervanines are most significant for their teratogenic effects; the most notable alkaloids were named cyclopamine (**12**) and jervine, both potent inducers of the congenital cyclopia monkey faced lamb syndrome reported in many flocks of sheep in the late 1950s in central Idaho. This cyclopic defect is induced in the sheep embryo during the blastocyst stage of development when the pregnant mother ingests the plant during the 14th day of gestation. Early embryonic death up to day 19 of gestation and other defects, such as limb defects and tracheal stenosis, occur when maternal ingestion includes days 28–33 of gestation (Keeler and

much less likely to induce birth defects. The structure–activity relationship is very important in potency to produce birth defects. It is now known that this structure–activity relationship is key in the mechanism of action, which is the inhibition of the sonic hedgehog signaling pathway (Gaffield and Keeler, 1996). This sonic hedgehog gene pathway and the subsequent downstream regulation of other genes' expression have been implicated in numerous cancers, birth defects, and other anomalies. The toxin cyclopamine has become a significant tool in the study of this very complex sonic hedgehog pathway. Clinical trials have been proposed and studies are ongoing to further identify the hedgehog complex of genes and to understand its mechanism and function in formation and growth of numerous cancers, childhood birth defects, and manipulation of regulatory pathways.

Clinical signs of poisoning are most likely caused by the neurotoxic cevanine alkaloids present in most species of *Veratrum*. Typical signs begin with excess salivation with froth around the mouth, slobbering, and vomiting progressing to ataxia, collapse, and death. The elimination half-life of cyclopamine in sheep is approximately 1.1 h (Welch *et al.*, 2009b). Consequently, 8 h after consuming *Veratrum*, greater than 99% of the toxins should be eliminated from the animal.

## Prevention and treatment

Control of *Veratrum* is relatively easy with herbicides such as broad-leaf herbicides, and long-term control has been demonstrated. The teratogenic effects of *Veratrum* can be avoided by keeping sheep and other livestock species off pastures containing the plants during the first trimester of pregnancy. Observation of toxicoses in the field is rare unless herders move the animals soon after exposure. The neurological signs, which are likely produced by the cevanine alkaloids (both *Veratrum* and *Zigadenus* spp.), can be treated with atropine to improve the cardiovascular output. Activated charcoal to adsorb toxins and administration of picrotoxin to improve respiration have been recommended (Burrows and Tyrl, 2001).



Stuart, 1987). The solanidine alkaloids are also found in many *Solanum* spp. and are toxic and teratogenic. The cholestanes have been used as hypotensive drugs but are

## BRACKEN FERN (PTERIDIUM AND AQUILINUM)

### Distribution and habitat

The bracken fern family is worldwide in distribution and includes approximately 20 genera and more than

400 species. Although most species described are found in the tropics, there are 4 genera and 6 species described in North America (Burrows and Tyrl, 2001). The bracken fern most associated with toxicoses in the United States is *Pteridium aquilinum* (Kuhn), which is distributed throughout North America. It is reported to be one of the most widespread species of vascular plants, only exceeded in geographical range by a few annual weeds. Four varieties of *P. aquilinum* have been described: var. *pubescens* (western bracken fern), found throughout the western United States; var. *caudatum* (lacy bracken), restricted to southern Florida; var. *latiusculum* (eastern bracken), distributed throughout the eastern United States; and var. *pseudocaudatum* (tailed bracken), located mostly in the southeastern quarter of the United States.

Western bracken grows best in rocky, gravelly, well-drained soils in dry, open woodlands to semishaded habitats. Extensive stands frequently grow along pasture edges, in logged areas, or where fires have opened up the canopy. Moisture needs are modest in comparison with those of many of the ferns, and good soil drainage is important.

## Description

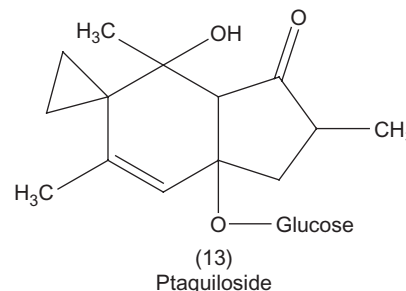
Bracken plants are deciduous and grow from brown to black woody rhizomes, forming large, often dense patches. The leaves emerge from erect fronds and are pinnately compound, scattered, erect, coarse, narrowly or broadly triangular, to 2 m in height. Fronds (leaves) are pinnules (ultimate segments), oblong, entire in the apices of the pinnae, and lobed toward the stalk. Reproduction is by spores produced in sporangia lining the undersurface margins of the photosynthetic fronds; when reproductive, they are covered by the narrow recurved edge of the leaf (Burrows and Tyrl, 2001).

## Toxicology

Toxicity of bracken fern was first recognized in the 1800s and described in horses as a neurological condition. Contaminated hay was believed to be the cause, and the condition was described in the United Kingdom and Pacific Coast states of the United States (Taylor, 1990). Early research determined that 20–25% bracken for 3 or 4 weeks would induce a neurological disease followed soon after by death. Although the neurological condition is mostly described in horses, low hematocrits and reddish-brown urine have also been described in suspected cases of bracken poisoning.

Bracken causes a wide range of syndromes that have been described in livestock, including thiamine deficiency in monogastrics, acute hemorrhagic disease

associated with bone marrow aplasia and ulceration of the upper GI tract, “bright blindness” progressive retinal degeneration, and neoplasia of the urinary bladder and upper digestive tract. The major toxin is the sesquiterpene glucoside, ptaquiloside (13). Other toxins, carcinogens, and mutagens may also be implicated in the disease conditions.



People have consumed the rhizomes or croziers of bracken fern as a traditional food or out of necessity, and the toxin is transferred through milk of cows grazing the plant. Epidemiological evidence suggests that some cancers in humans probably result from primary or secondary consumption of the carcinogens. Ptaquilosides form adducts with DNA, binding to certain base sequences, resulting in mutated codons associated with known oncogenes. This hypothesis has been proposed in the pathogenesis of the disease.

Syndromes of bracken poisoning are well recognized in livestock. Many factors must be considered in the genesis of the disease, such as quantity consumed, phenological stage of the plant, time of year, consumption rate and length of time consumption occurs, animal species, age, and sex of the animal. The disease conditions described include thiamine deficiency in monogastrics, acute hemorrhagic disease, bright blindness, enzootic hematuria, and small intestine carcinoma. These conditions have been experimentally produced by feeding bracken fern and ptaquiloside to livestock species and rodent models. Bracken fern feeding studies in rodents have produced neoplasms in the ileum, urinary bladder, mammary glands, and lungs of rodents.

The toxin ptaquiloside was isolated, characterized, and the structure published in the early 1980s (Saito et al., 1990). Different species and varieties of *Pteridium* have been compared for ptaquiloside concentration, and all examined to date contained ptaquiloside. Large variations in concentration were demonstrated between locations, altitude, season, etc., suggesting a genetic component or another factor, such as endophyte-produced compounds. These differences were maintained when bracken fern rhizomes were transferred to a greenhouse and soils were changed; thus, the ptaquiloside differences



were still evident for the next 3 years. Ptaquiloside concentrations are highest in young growing parts (i.e., the tips of the croziers and immature fronds), whereas concentrations diminish as the plant matures and as samples are taken from more mature pinnae and toward the base of the lamina. In all samples of rhizome, the apices of the fronds or primordia taken from below the soil surface had no ptaquiloside; however, very immature croziers only a few centimeters above the surface showed the presence of the toxin. The emergence of the crozier apices and exposure to light apparently influence the biosynthesis of the toxin. The spores have not been shown to contain ptaquiloside, but they have caused cancer and formed DNA adducts, suggesting that carcinogenic or mutagenic derivatives are present. Ptaquiloside and other ptaquiloside-like compounds with carcinogenic activity have been isolated from other ferns from the genera *Histiopteris*, *Cheilanthes*, *Cibotium*, *Dennstadtia*, *Hypolepis*, *Pteris*, and *Pityrogramma* (Saito *et al.*, 1990).

Although bracken fern poisoning occurs in the United States, the most serious risk is in countries such as Australia, New Zealand, Japan, and the United Kingdom, where bracken is more prevalent and utilized for human food or animal feed. Epidemiology studies have associated increased esophageal and gastric cancers with direct ingestion, secondary ingestion (i.e., milk from cows grazing bracken), or living in bracken fern-infested areas. Bracken fronds steeped in water or treated with wood ash or sodium bicarbonate have reduced toxicity, but this process only reduces the concentration and does not eliminate the toxins.

Lesions in horses poisoned by bracken fern are indicative of thiamine deficiency and include congestion of the brain, a swollen and edematous cerebrum grossly, and necrosis of some neurons microscopically. Acute hemorrhagic disease in cattle is characterized by extensive hemorrhage of the mucous membranes and subcutaneous hemorrhage and edema.

Pathology of the bracken-induced enzootic hematuria includes desquamated and proliferative bladder epithelium, and areas of vascularized epithelial proliferation appear as polyploid, papillary, or fungoid reddened foci. Microscopically, columns of transitional epithelium infiltrate into the lamina propria along with mononuclear cells. Neoplasia, when it occurs, is most commonly the noninvasive papillomatous type, but other papillary types of transitional cell carcinoma, squamous cell carcinoma, adenocarcinoma, or hemangiomas may also develop (Burrows and Tyrl, 2001).

## Treatment

Bracken-induced thiamine deficiency in horses is treatable with administration of thiamine parenterally at



**FIGURE 77.9** Narrow leaf (left) and broad leaf (right) milkweeds (*Asclepias* spp.) represented.

0.5–1g, followed by decreasing doses during the next few days. Symptomatic care with good feed and fresh water accompanied by administration of a laxative but not mineral oil is helpful. In ruminants, the bone marrow suppression and deficiency of blood platelets and neutrophils is best treated with antimicrobials to counteract any bacterial infection that might occur because of diminished immune function. Good veterinary care, symptomatic treatment, clean water, and quality feed in a quiet, clean environment are recommended.

## MILKWEEDS: ASCLEPIAS SPP.

### Description

Milkweeds are classified into two broad groups: (1) narrow-leaved, with narrow, linear, lanceolate leaves, and (2) broad-leaved, with leaves approximately 4cm wide throughout much of their length (Figure 77.9). There are more than 150 species of *Asclepias*, of which 108 occur in North America. Milkweeds are perennial, summer or early autumn flowering herbs with a milky latex-like cream in the stems. Of the many species found in North America, several are important toxicologically and are represented in Table 77.12. The stems of the plants are erect and range from 4 to 150cm tall; leaves are opposite or whorled. Flowers are of various colors, umbellate

TABLE 77.12 Milkweeds: *Asclepias* spp., common name, distribution, habitat, and toxin

Species	Common name	Distribution	Habitat	Toxin; content <sup>a</sup>
<b>Narrow-leaved milkweeds</b>				
<i>A. labriformis</i> Jones	Labriform milkweed	Southeastern UT	Along old streambeds in sandy soils	Cardiac glycosides, digitoxin; very high
<i>A. verticillata</i> L.	Eastern whorled milkweed, spider milkweed	From TX to MI, east to FL and MA	Dry open areas	Cardenolides; very low
<i>A. subverticillata</i> Vail	Western whorled milkweed, horsetail milkweed	Western KS and OK, UT, AZ, TX and into Mexico	Dry plains and foothills, spreads rapidly along waterways and canals	Cardenolides; very low
<i>A. pumila</i> Vail	Low whorled milkweed, plains whorled milkweed	East of the Rockies from TX and NM north to southeast MT and southwest ND	Small patches in draws and ravines	Cardenolides; very low
<i>A. mexicana</i> Cav.	Mexican whorled milkweed	Southern TX to central Mexico	Open areas and dry soils	Cardenolides; unknown
<i>A. asperula</i> Woodson	Antelope horn milkweed	KS, AR to NV and AZ	Open areas and dry soils	Cardenolides; very high
<i>A. fascicularis</i> Decne.	Mexican milkweed	CA, western OR through eastern WA, ID and NV		Cardenolides; very low
<i>A. brachystephana</i> Engelm.	Short-crown milkweed	West central TX, southern AZ into NM and Mexico		Cardenolides; very high
<i>A. subulata</i> Decne.	Desert milkweed, yamate, ajamete	Southern CA, AZ, west coastal region of Mexico		Cardenolides; very high
<b>Broad-leaved milkweeds</b>				
<i>A. eriocarpa</i> Benth.	Woolly pod milkweed	CA	Dry soils	Cardenolides; very high
<i>A. latifolia</i> Brit.	Broadleaf milkweed	KS, CO to TX and AZ	Dry plains	Cardenolides; high
<i>A. speciosa</i> Torr.	Showy milkweed	MN south to MI and TX; west to CA	Prairies and open spaces	Cardenolides; intermediate
<i>A. syriaca</i> L.	Common milkweed	Widely distributed in central and eastern U.S.	Open areas and along roadsides	Cardenolides; intermediate
<i>A. incarnata</i> L.	Swamp milkweed	IN	Cool damp soils	Cardenolides; very low
<i>A. vestita</i> Hook and Am.	Woolly milkweed	Central and south western CA		Cardenolides; very high
<i>A. viridis</i> Walter	Green milkweed, spider milkweed	South-central states, FL		Cardenolides; high

Adapted from Burrows and Tyrl (2001) and Knight and Walter (2001).

<sup>a</sup>Cardenolide content mg/g plant from <0.25 (very low) to >4 (very high).

clusters or solitary, and terminal or extra-axillary. Numerous seeds are contained in an inflated milkweed-type pod and are flat and large, with each bearing a tuft of long, silk-like hairs for wind distribution.

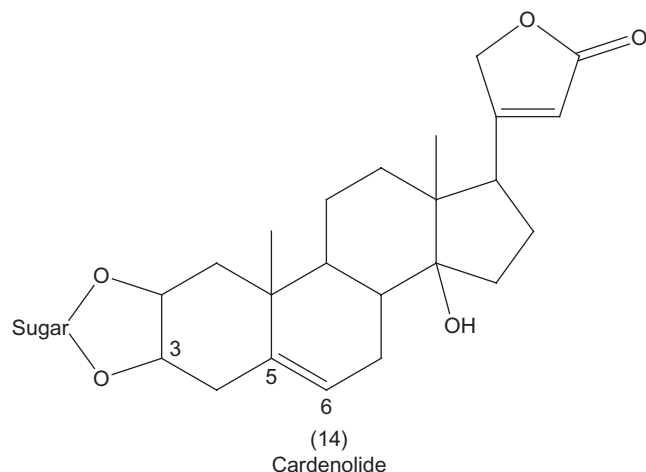
## Distribution and habitat

Milkweeds are adapted to a broad range of habitats and environmental conditions and are found in most plant communities. Occupied habitats include prairies, woodlands, open areas, rocky plains, sand dunes, swamps, marshes, seeps, canyon bottoms, dry washes, desert lands, and waste lands. Most species are weedy and form small to large patches often along roadsides, in disturbed areas, or in overgrazed pastures.

## Toxicology

Some species of milkweeds have been used as medicines, food, or a source of poison. Most species contain a mixture of steroidal glycosidic cardenolides (**14**) that are toxic and induce two syndromes – one with GI/cardio-toxic effects and the other with neurological effects. The early literature suggested that the broad-leaved group was cardiotoxic, whereas the narrow-leaved type was neurotoxic. However, most narrow-leaved species have now been shown to also contain cardiotoxins (Ogden *et al.*, 1992).

Historically, naturalists observed as early as the mid-1800s that birds avoided eating certain butterflies, such as the monarchs, whose larvae fed on milkweeds. It was later learned that the feeding larvae were able to



concentrate some of the cardenolides that were potent emetics and thus created an effective defense against herbivory. As of the late 1980s, 27 species of milkweed were recognized as common feed for butterfly larvae, and the most toxic species were often preferred. Milkweeds are also host to many other insect species.

The basic structure of the series of identified cardenolides is a 23-C steroidal backbone with a five-membered, singly unsaturated lactone ring at the C-17 position, a hydroxyl group at C-14, and methyl groups at C-10 and C-13. Glycosidic linkage usually occurs at the C-3 or C-2 positions and may include glucose, rhamnose, or thevetose as the sugars. Additional functionalities attached to the backbone further influence solubility and binding, thus increasing or decreasing toxicity. The cardenolides inhibit  $\text{Na}^+/\text{K}^+$ -ATPase, and the structure-activity relationship is believed to reside in the unsaturated lactone ring at C-17 and the hydroxyl group at C-14 (Joubert, 1989).

Sheep, goats, cattle, horses, and domestic fowl have been poisoned with milkweeds. Clinical signs usually begin with depression, weakness, and labored breathing accompanied by ataxia and loss of balance, progressing to recumbency, seizures, and death. The order of clinical signs is dependent on the type of animal affected, the toxicity of the plant, the time of ingestion, and the amount of the toxin consumed. Clinical effects appear within a few hours of ingestion, and death may follow within 1 to a few days later in fatal cases.

## Treatment and control

There are two syndromes of milkweed poisoning – a neurological one and a cardiac one. Treatment for the cardiotoxic signs is approached similar to that of digitalis glycoside toxicity – that is, activated charcoal, atropine for atrioventricular block, and/or antiarrhythmic drugs. There is no specific treatment for the neurological

syndrome except symptomatic treatment to control seizures and supportive therapy. A field test with a detection threshold of 0.057% is available for detecting cardenolides in the latex of milkweeds (Sady and Seiber, 1991). Knowledge of the milkweed species in the pasture, stage of plant growth when consumed, and history of grazing and clinical effects are all important in developing a diagnosis and eliminating other causes.

In most cases, knowledge that the plant exists in one's pasture and prevention of ingestion is the best way to avoid poisoning. Purchase of weed-free hay to avoid poisoning via contamination is also important. Know where the hay comes from, and always be alert as to the content of the hay. Control of milkweeds can be accomplished with herbicides such as 2,4-D, picloram, amitrole, or glyphosate, depending on the other forage it has infested and the circumstances. Always follow the manufacturer's recommendations.

## NIGHTSHADES

The nightshade family comprises more than 80–90 genera and more than 2300 species worldwide. Some of the more common poisonous genera are *Brugmansia*, *Brunfelsia*, *Capsicum*, *Cestrum*, *Datura*, *Hyoscyamus*, *Lycium*, *Lycopersicon* (tomato), *Nicotiana* (tobacco), *Solandra*, *Solanum*, *Nicandra*, and *Physalis*. Some common nightshade species are listed in Table 77.13. In the United States and Canada, the nightshades can be annual, perennial, or shrubs with alternate, simple or compound leaves, axillary inflorescences, radially symmetrical flowers composed of five free sepals, five free petals, and usually five stamens with anthers grouped conically about the stigma and berry-like fruits of variable colors.

## Toxins

The nightshades have a variety of toxins known to affect people and animals, including the tropane alkaloids, which affect the autonomic nervous system by blocking the action of cholinesterase (Knight and Walter, 2001). This results in accumulation of acetylcholine and subsequent inhibition of the parasympathetic nervous system, causing dry mouth, decreased intestinal motility, dilated pupils, and tachycardia. The tropane alkaloids also affect the GI tract, causing colic in horses, constipation, and/or hemorrhagic diarrhea. The *Nicotiana* spp. contain neurological toxins including the pyridine and piperidine alkaloids that are responsible for toxicoses and teratogenesis. Some species of *Solanum* cause a neurological disease in cattle, sheep, and goats characterized by loss of equilibrium,

TABLE 77.13 Nightshade species with known links to toxicosis in humans or animals: common names, distribution, and toxins

Species	Common name	Distribution	Toxins
<i>Solanum elaeagnifolium</i>	Silverleaf nightshade	Southern U.S.	Solanine and solanidine
<i>S. nigrum</i>	Black nightshade	Throughout North America	Tropane and glycoalkaloids
<i>S. dulcamara</i>	Bittersweet; climbing nightshade	Eastern half of U.S.; areas of OR, WA, NV, CA, UT and WY	Glycoalkaloids
<i>S. rostratum</i>	Buffalo bur	Central U.S. to north and south borders	Glycoalkaloids
<i>S. triflorum</i>	Cutleaf nightshade	Central U.S. to north border	Glycoalkaloids
<i>S. americanum</i>	Huckleberry; wonderberry	Southern U.S.	Glycoalkaloids
<i>S. physalifolium</i>	Tropical soda apple	Throughout U.S.	Glycoalkaloids
<i>S. sarrachoides</i>	Hairy nightshade		Glycoalkaloids
<i>S. carolinense</i>	Horse or bull nettle	Eastern half of U.S.; west coast	Tropane alkaloid solanine
<i>S. tuberosum</i>	White or Irish potato	Worldwide	Glycoalkaloids
<i>Datura wrightii</i>	Sacred datura	Southwestern U.S.	Tropane alkaloids
<i>D. stramonium</i>	Jimson weed; thorn apple	Southeastern half of U.S.; west coast	Tropane alkaloids
<i>Nicotiana tabacum</i>	Cultivated or burley tobacco	Southern U.S.	Pyridine alkaloids (nicotine)
<i>N. glauca</i>	Tree tobacco	Southern U.S.	Piperidine alkaloids
<i>Capsicum annum</i>	Green or chili pepper	Cultivated in U.S.	Capsaicinoids
<i>Cestrum parqui</i>	Willow-leaved jessamine	Southern Gulf states	Glycoside of vitamin D
<i>Atropa belladonna</i>	Deadly nightshade; belladonna	Cultivated as an ornamental	Tropane alkaloids
<i>Hyoscyamus niger</i>	Black henbane	Northern U.S.	Tropane alkaloids, calystegins
<i>Physalis virginiana</i>	Ground cherry	Plains states and eastern U.S.	Glycoalkaloids, calystegins
<i>P. lobota</i>	Chinese lantern	Southern regions; cultivated throughout U.S.	Glycoalkaloids
<i>Lycopersicon esculentum</i>	Tomato	Worldwide	Glycoalkaloids

Adapted from Burrows and Tyrl (2001) and Knight and Walter (2001).

tremors, ataxia, collapse, opisthotonus, seizures, and death. *Solanum malacoxylon* and *Cestrum* spp. contain vitamin D-like compounds that cause abnormal calcium absorption and metabolism resulting in calcified tissues, lameness, and weight loss. Green potatoes, sprouts, and vines contain steroidal glycoalkaloids that are both toxic and teratogenic. Table 77.13 provides a limited overview of selected species, their distributions, and their toxins (Burrows and Tyrl, 2001; Knight and Walter, 2001).

## Clinical signs

The toxins in the Solanaceae family contribute to various clinical effects depending on the amount of plant/toxin ingested, the plant species eaten, and the animal species consuming the plant. Signs range from mild digestive upset to severe colic in horses when contaminated hay is fed; neurological dysfunction, seizures, and death in sheep and cattle; big head and calcification of the blood vessels; and teratogenesis.

## Treatment

Animals showing severe neurological signs, such as tremors, ataxia, and dilated pupils, may be treated with physostigmine. Oral activated charcoal as an adsorbent

may be effective if administered in a timely manner. However, most animals will recover if treated symptomatically and if the animals are not overly stressed.

## KNAPWEEDS: CENTAUREA SPP.

The knapweeds are a large group with primarily noxious, invasive characteristics. Although this genus is not a great risk for livestock producers, a serious disease of horses called nigropallidal encephalomalacia warrants its inclusion in this chapter. There are 450–500 species of *Centaurea*, and 29 species have been described in North America (Burrows and Tyrl, 2001). Most of these have been introduced and have had a huge negative impact on rangelands in the western United States. Although most species are opportunists and will aggressively invade rangelands, especially those that have been overgrazed, burned, or disturbed, only 2 species are of any toxicologic significance – *Centaurea repens* (Russian knapweed) and *C. solstitialis* (yellow star thistle).

## Habitat and distribution

Yellow star thistle is most abundant in the western United States from central California north through



Oregon, Washington, and Idaho. Smaller invasions are reported in many states east of the intermountain region, and although these have not received the attention of the larger invasions in the west, they have the potential to rapidly spread under the right environmental conditions (Panter, 1991; Burrows and Tyrl, 2001; Knight and Walter, 2001). Russian knapweed has invaded very large areas of the intermountain region of the western United States and the Great Plains. Again, smaller populations have invaded areas of the Midwest and northeastern United States and have the potential to expand rapidly. Although the aggressive nature of these species threatens rangelands and prohibits optimum utilization, a greater threat is the risk to sensitive or threatened native plant species and the balance of plant biodiversity.

## Toxicology

The compounds isolated from knapweeds include a large class called sesquiterpene lactones. Although the putative toxin causing the neurological disease in horses has not been specifically identified, six of these compounds have been screened for cytotoxicity in an *in vitro* neuronal cell bioassay. The rank order of activity is repen > subulateolide > janerin > cynaropicrin > acroptilin > solstitialin (Riopelle and Stevens, 1993). Toxicity of solstitialin A-13 acetate and cynaropicrin to primary cultures of fetal rat substantia nigra cells has been demonstrated. These sesquiterpene lactones are quite unstable, and it has been hypothesized that they are precursors to the ultimate neurotoxin. Also, there are aspartic and glutamic acids present in these plants, and they possess neuroexcitatory properties.

## Clinical signs

Thus far, only yellow star thistle and Russian knapweed have been implicated in toxicoses in the United States and only in horses. Apparently, ruminants are not affected, and the *Centaurea* spp. may be useful forage for sheep and goats. However, in other countries, toxicoses in ruminants have been reported. For example, in South Africa, *C. repens* fed to sheep at 600 g dosages for 2 days caused an acute digestive upset and pulmonary edema and ascites. In Azerbaijan, *C. repens* is reported to cause a neurological disease in buffalo similar to that which has been described in horses. However, no neuropathology similar to that seen in horses was observed in the buffalo.

Toxicity generally occurs in summer and fall when forage is depleted and horses are forced to graze less palatable species. Ingestion often occurs for several months or more before an abrupt onset of neurological dysfunction is observed. Impaired eating and drinking are often the first observable signs. Depression and hypertonicity

of the lips and tongue follow, and a constant chewing may be observed, hence the name "chewing disease." Abnormal tongue and lip postures may be observed, and other neurological signs include locomotor difficulties such as aimless walking, drowsy appearance, and inactivity with the head held low. The neurological disease is considered permanent, and although some improvement may be seen, difficulty eating and drinking may preclude long-term recovery. Often, the disease progresses to dehydration, starvation, and bizarre behavior including submergence of the head in water to allow water to flow into the esophagus or lapping water like a dog. *Centaurea repens* appears to be more toxic than *C. solstitialis*, but prolonged ingestion is required by both before disease appears. The amount of plant ingested to induce the clinical effects is reported to be 60% or more of body weight for *C. repens* and 100% or more of body weight for *C. solstitialis*. Intermittent grazing can prevent disease, indicating that there is not a cumulative effect but, rather, a threshold must be exceeded before neurological signs are observed (Cordy, 1978). Once neurological signs are observed in horses, prognosis for recovery is poor and euthanasia should be considered.

## Pathology

The lesions are very specific and limited to the globus pallidus and the substantia nigra (nigropallidal encephalomalacia), where distinct pale yellowish to buff-colored foci or softening and cavitation are seen (Cordy, 1978). The lesions are typically bilateral and symmetrical. This specificity of the lesions for the basal ganglia has prompted more investigations into unraveling the mysteries of human diseases associated with dopaminergic pathways, such as Parkinson's or Huntington's disease, and tardive dyskinesia. This disease in horses is often called equine parkinsonism. This unusual disease is manifest by an almost immediate onset after prolonged ingestion, suggesting an all-or-none type of acute neurological crisis. The lesions develop quickly and completely, and progressive stages of degeneration rarely occur except for some changes in the adjoining neurons adjacent to the necrotic foci in the globus pallidus and the pars reticularis of the substantia nigra (Cordy, 1978). Microscopically, there is extensive necrosis of neurons, glia, and capillaries within sharply defined margins of the involved brain centers. Occasionally, lesions may be observed in the gray and white matter of the brain.

## Prevention and treatment

Good veterinary care and supportive therapy including good feed, easy access to water, supplemental

vitamins, and good nursing care is essential for survival. Treatment of the disease once it is manifest is not generally successful. However, in Argentina, affected horses have been treated with glutamine synthetase and a bovine brain ganglioside extract given daily intramuscularly for 1 month with some success. When animals are first observed grazing *Centaurea* spp., they should be immediately removed to better pastures. Prevention of the disease is easily accomplished by knowing the plants that exist in one's pastures, by providing good quality and adequate amounts of forages and feed, and by frequent observation of one's animal's grazing patterns and behavior.

Control of plant invasion by good range/pasture management to prevent overgrazing and loss of other competitive grasses and forbs is important. Herbicide control is quite easily accomplished with broadleaf products including 2,4-D, dicamba, and picloram applied according to label. These plants are prolific seed producers, and follow-up treatment is required to eliminate the populations. Seeds are often distributed through contaminated hay or other feed sources, and initial populations often start near feed bunks and spread from there. Because of their morphology, size, and parachute-like structures, seeds are easily spread by wind and water. Understanding one's weeds and close monitoring of populations will help in the control of these highly invasive species.

## CONCLUSIONS

Even with our ever-increasing knowledge about poisonous plants and their toxins, poisonings continue to occur, some catastrophic, on livestock operations. Poisoning in humans and companion animals from toxic plants also continues to be a significant risk, especially to pets and children. As the influx of small-acreage farmers onto native rangelands increases and ranchettes become more common, the potential risk for poisonous plant problems increases. Lack of understanding and increased grazing pressure on these small acreages often contribute to the consumption of toxic plants by animals. In some cases, novel or unusual animal species are exposed to unfamiliar forages, further contributing to potential poisonings. Plant poisonings will undoubtedly also increase in wild-life populations as humans continue to encroach on their native ranges and interrupt their migratory pathways.

The following basic concepts can help reduce risk of poisoning:

- 1 Understand and recognize the plants on your range or pastures and know the potential hazards of grazing

where poisonous plants grow. Know the conditions under which poisoning may occur.

- 2 Do not introduce unfamiliar animals onto ranges where poisonous plants may present a hazard.
- 3 Avoid introducing animals to poisonous plant-infested ranges when adequate, good-quality forage is not available.
- 4 Do not throw grass, shrub, or tree clippings into paddocks where animals reside (yew clippings are a common cause of poisoning in many animals).
- 5 Provide free access to fresh water and minerals/salt.
- 6 Do not overstock the range or pastures.
- 7 Avoid bedding, lambing/calving, watering, salting, or unloading hungry animals near poisonous plant populations.
- 8 Avoid excess stress to those animals showing clinical signs of poisoning, and contact your veterinarian.
- 9 If economically feasible, control poisonous plants through hand grubbing, mechanical clipping, or herbicide treatment.

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## Poisonous plants of Europe

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### INTRODUCTION

European flora is less rich in species and less dangerous than the American and African flora; however, the number of plant poisons is considerable.

Poisoning of livestock and companion animals by plants is a sporadically occurring clinical problem. Most plants cause poisoning only when they are eaten. The possibility of plant poisoning is usually neglected in a differential diagnosis because plant poisoning is uncommon. In intensive rearing of animals such as swine or poultry, contact of the animal with a toxic plant is unlikely. Also, the maintenance in confinement of some semipermanent stall dairy cattle and young beef cattle minimizes this risk. The common uses of herbicides destroy some poisonous plant species. However, in many cases, it must be determined whether animals may be in contact with toxic plants either in the pasture or by means of poisonous plants mixed with forage and distributed in conserved forage.

Plant poisoning in small animals is usually accidental. Accidents can be prevented by understanding the conditions in which poisoning may be expected to occur and then taking steps to prevent its occurrence. Moreover, problems can occur with animals in ornamental, garden, and natural environments and homes. Pets are usually the victims of this type of accident. Many veterinarians are not familiar with plants found in and around the home that may be potentially harmful if ingested; therefore, they are responsible for being prepared to deal with plant poisoning should it occur. Many house and garden plants grown in Europe contain chemical substances in sufficient quantities that lead to toxic effects in animals. Dogs and cats are not herbivores and infrequently consume large

quantities of plant materials. It is recognized, however, that they do occasionally ingest plant materials, especially grass. When conditions are favorable, both dogs and cats will consume other herbaceous materials that cause toxic conditions. Although plant poisoning of adult dogs and cats is unlikely, puppies often taste anything they encounter and are the principal candidates for accidental ingestion of toxic plant materials.

Occasionally, a client may observe a pet consuming a fruit or leafy material. A frantic call to the veterinarian may prove fruitless if the plant cannot be identified by visual description. The amount consumed is of importance. Rarely will a single bite of any plant prove to be lethal. Fortunately, most plants do not cause toxicity, or they may cause mild to moderate gastrointestinal irritation. A few plants in certain localities, regions, or countries can produce serious toxicity, including oleander (*Nerium oleander*) and castor beans (*Ricinus communis*).

Household pets are exposed to the same harmful substances as young children. Puppies and kittens, like children, seem to have a predilection to ingest attractive foreign materials, such as parts of house and garden plants (berries, fruits, seeds, and leaves). Both older and immature animals may suffer from boredom or behavioral abnormalities or may simply be investigating a new addition to the environment, all of which may lead to ingestion of poisonous plant material.

Small pet animals are at greatest risk with exotic plants. They are not herbivores, and as a result, they never develop detoxification mechanisms for plants. By contrast, most herbivores have co-evolved elaborate detoxification mechanisms to cope with the thousands of alkaloids, glycosides, amines, and resinoids found in plants that they usually eat. When animals and plants evolve together, a

commensal relationship often develops. Such relations are interrupted when plants are introduced into new environments, exposing the resident animals to substances for which they lack detoxification mechanisms.

Finally, many poisonous substances found in plants are also used medicinally as drugs. Cardioactive glycosides, atropine, and acetylcholine are three examples. Poisoning from plant material presupposes consumption of sufficient quantity of one or several active ingredients that in turn can act synergistically. The toxic dosage varies with the species of the plant, the stage of plant growth, the part of the plant consumed (i.e., leaves, roots, etc.), the type of soil, and other environmental factors.

Plants can be roughly classified depending on which body system they affect. The pharmacology and toxicology of commonly found plants are known. However, even if a plant is suspected of causing an adverse outcome, without information about similar cases, toxicological analysis of biofluids, or pharmacological or toxicological effects of the plant, further investigation might prove difficult or impossible.

## Plant identification

Plant toxicity occurs consistently at the genus level, although species variation may alter toxicity. Consequently, accurate identification of the plant is necessary (genus and species), which often requires recognition of the scientific name and the plant parts by a qualified person. Usually, a family member knows the common names of household and garden plants, but the problem is that the same common names are used for many different plants (e.g., milk plant and milkweed). Some are hazardous to livestock, and other ornamentals may be hazardous to small animals. Still others are edible or innocuous (Fowler, 1981). For this reason, therapeutic decisions are sometimes based on imprecise common names. Currently, plant toxicology is changing; knowledge of poisonous plant names is important to ensure that the same name is universally applied. Both common names and scientific names are needed for communication with the public and for scientific exchange.

Ornamental plants are not easily identified botanically. Standard botanical keys are not very helpful. If the species of an ornamental plant is in question, it should be taken to a qualified person for identification. Botany or horticultural herbaria in museums, colleges, or universities are helpful resources for plant identification (Forsyth, 1968).

## Diagnosis of plant poisoning

The following information is usually required for diagnosis: region, time and amount of exposure, type of

plant, part of plant ingested, onset of symptoms, type of symptoms, and the presence of insecticides or herbicides. A formal diagnosis of poisoning must be made using a thorough history, clinical signs, and laboratory analysis of samples (Table 78.1). The owner may be able to provide data on what intoxication may be in the environment in which animals are rearing or living. However, owners are usually not aware of potential toxic plants that are present.

### Pet animals

The diagnosis of dog poisoning by plants or plant products is not always easy because symptoms are rarely characteristic. Their treatment is often disappointing. The clinical signs of poisoning produced by ornamental plants certainly were not noticed in the past, either because the clinical examination was lacking or because symptoms that presented in the poisoned animal were not sufficiently alarming to consult or notify veterinarians. The frequency and severity of poisoning are extremely variable. The toxicity of a given ornamental plant varies from one variety to another and from one organ to another, and it also depends on the state of maturity. Animals react differently depending on the species, age, health status before poisoning, their sensitivity to the plant and the amount ingested, and whether the plant has been chewed or not. It is difficult to make a diagnosis of poisoning by an ornamental plant in view of the few symptoms and lesions presented by a sick animal. It is essential to perform an accurate clinical examination and to detect pieces of plant identified in the stool or vomit to diagnose by checking if the symptoms correspond with those observed in similar cases in previous experiments. The symptoms and lesions are varied;

TABLE 78.1 Diagnosis of plant poisoning

#### General approach

History: After stabilization of vital signs. The questions addressed to the animal owner should be accurate.

Plant identification: Accurate identification of the plant is needed (genus and species) as well as recognition of the scientific name, common name, and plant parts.

Clinical signs (including odors).

Laboratory tests: Toxicant isolation, search for toxic plants (in the meadow or forage), and search of toxicant in animals (rumen and stomach).

#### Treatment

Decontamination, especially gastrointestinal tract: emesis, gastric lavage, activated charcoal, enemas, and laxatives/cathartics.

Elimination enhancement: Diuresis, urine acidification, and urine alkalization.

Antidotes.



they predominantly affect the digestive tract when the poisoning is benign and the nervous and cardiovascular systems when poisoning is serious. At necropsy of the intoxicated animal, gastroenteritis lesions are often observed. For the previous reasons, prevention must play an essential role. It is necessary to know the plants that are poisonous to pets and avoid planting them in kennels or maintaining them in the home. It is also necessary to ensure that animals receive a sufficient and balanced diet, corresponding to their specific needs, and that they have regular daily exercise. Finally, hazardous material, whatever its nature, should not be dragged on the ground because dogs, and especially infants, can be the first victims.

*Liliacea* spp. and *Araceae* spp. were the major plant families involved in poisoning; most of the cases were related to accidental ingestion of ornamental plants rather than wild plants, particularly during certain times of the year. Cats appeared to be more sensitive than dogs, probably due to the tendency to chew on plant leaves. *Convallaria majalis* and *Nerium oleander* were also commonly reported to cause severe and potentially lethal cardiac disorders, whereas *Ricinus communis* and *Datura stramonium* cases were also frequently observed (Gault *et al.*, 1995).

Small animal veterinarians must deal with a number of potentially poisonous, commonly occurring household and garden plants (Table 78.2). In many cases, the client cannot name the plant that the animal encountered in his or her house or property. As indicated, veterinarians should familiarize themselves with the most potentially toxic plants and their clinical signs and any naturally occurring dangerous plants in their area (Flood and Fitzgerald *et al.*, 2006).

Livestock

Livestock can be poisoned by many different plant species, but an analytically confirmed diagnosis is not always easy, and most cases are only suspected following necropsy and identification of leaves, stems, or pods in the rumen. A diagnosis of plant poisoning can rarely be made by the clinical syndrome alone. Signs accompanied by a history of exposure to the plant are usually necessary. If a client presents a short-haired hunting dog with trembling and salivation and explains that the dog has been in a field trial all day, stinging nettle toxicity should be an obvious diagnostic possibility.

Judicious questioning of the client may be necessary when a poisonous plant is suspected. The client may not know the plants in the home or garden if a spouse or other family member is the gardener, and further inquiry is necessary to establish the presence of a suspected plant in or around the home.

Conditions conducive to plant poisoning

Cross-breeding, environmental conditions, and seasonal variation in toxin content may also alter toxicity outcome. Although plant poisoning of adult dogs and cats is possible, puppies may eat anything they encounter and are prime candidates for accidental ingestion of toxic plant material. Kittens are less likely than puppies to ingest such foreign material but occasionally will do so (Fowler, 1981).

Caged birds present special problems. Psittacines (budgerigars, cockatiels, parrots, and macaws) are generally seed-eaters. As a rule, they are fed seeds from a

TABLE 78.2 Household plants

Family	Genus/species	Clinical presentation
Amaryllidaceae	<i>Amaryllis</i> , <i>Clivia</i> , <i>Crinum</i> , <i>Hippeastrum</i>	Digestive disorders (vomiting and diarrhea).
Apocynaceae	<i>Allamanda cathartica</i> , <i>Dipladenia sanderi</i>	Skin (dermatitis) and digestive disorders.
Araceae		
Dieffenbachia	<i>Dieffenbachia sanguine</i> , <i>D. picta</i>	Keratoconjunctivitis, edema of the eyelids, and corneal ulceration; reactions of mouth (lips, tongue, palate, and pharynx), esophagus, and stomach injuries (vomiting and diarrhea). The cat is particularly sensitive.
Other dangerous Araceae	<i>Alocasia</i> spp., <i>Anthurium</i> spp., <i>Arisaema</i> spp., <i>Caladium</i> spp., <i>Monster</i> spp., <i>Philodendron</i> spp., <i>Xanthosoma</i> spp., <i>Zantedeschia aethiopica</i>	They can cause similar disorders to <i>Dieffenbachia</i> and sometimes dermatitis as a result of animal contact with the plant.
Ericaceae	<i>Rhododendron obtusum</i> , <i>R. simsii</i>	Vomiting, diarrhea, violent equilibrium changes and convulsions, paralysis, and dyspnea; coma and death (at high doses).
Euphorbiaceae	<i>Euphorbia pulcherrima</i>	Irritating to skin and projected into the cornea; local disorders and vesicular dermatitis, conjunctivitis; stomatitis, vomiting, and diarrhea.
Liliaceae	<i>Tulipa</i> sp., <i>Hyacinthus</i> spp.	Vomiting with gastroenteritis.
Moraceae	<i>Ficus elastic</i> , <i>F. lyrata</i>	Similar to those caused by Euphorbiaceae and also urinary signs.

commercial source, and the likelihood of poisoning is remote. Sometimes a well-meaning owner collects and feeds wild seeds, which can pose a slight hazard if the person is unable to identify all of the plants from which the seeds are collected (Fowler, 1981).

Higher concentrations of any poisonous substance are frequently found in seeds than in the rest of the plant. Thus, seed-eaters such as psittacine birds are at particular risk when such plants are the source of food.

Birds that are allowed to fly free in the home or that escape from a cage may peck at and ingest poisonous houseplants.

## Predictive and toxicity factors

### Age

By nature, puppies and kittens continually explore with their mouths. Teething irritation may be relieved by chewing on objects in the environment. Any available plant materials, such as seeds, pits, bulbs, branches, and even leaves, may be chewed and ingested. Puppies and kittens have low activities of the hepatic biotransformation enzymes as well as low levels of conjugating moieties. A poorly developed blood-brain barrier, weak biotransformation activity, and immature mechanisms for excretion combine to make puppies very vulnerable to toxic effects of poisonous plants. Owners should be informed of the necessity to “pet proof” the surroundings of young animals by removing or preventing access to hazardous substances, including toxic plants (Fowler, 1981).

### Species

Some species are very susceptible to toxic plants, whereas others are not susceptible. For example, rabbits are insensitive to the effects of *Galega officinalis*, and certain species of birds can consume *Cicuta* spp. without toxicity occurring.

### Boredom

Both older animals and immature animals may suffer from apathy. Dogs, in particular, have problems resigning themselves to continuous confinement compared to others that have unlimited freedom. A dog with nothing to do may resort to aberrant behavior, such as ingestion of any available plant material(s). If these plants are poisonous, toxicity may result. Clay (1977) described three instances of dogs poisoned by plants within their confined space.

### Changes in surroundings

New objects placed within the environment of a pet are novel and subject to a thorough investigation. During the holiday season, new plants are often brought into

the home, and some of them are potentially dangerous. When a family moves into a new home or goes on vacation with its pet(s), new plants are often encountered.

Pets used to “star billing” in a household may feel neglected when a new baby arrives and receives all the attention. In such circumstances, many pets resort to pica. Illness of an owner may require “pet sitting” by others and thus initiate the same response (Fowler, 1981).

### Behavioral abnormalities

Behavioral abnormalities are increasingly being recognized as specific clinical entities. A few behaviorist-trained veterinarians are beginning to unravel some of the complex interrelationships of pet behavior and disease. Ingestion of foreign materials is one such manifestation of deranged behavior. Plant poisoning may be a consequence of such behavior. The veterinary profession needs to distinguish the many minor exposures from the few serious ones and to treat those toxic exposures appropriately. Such a task may be difficult because the plant involved is not easily identified. Even when it is known, predicting toxicity is difficult because of variation in plant toxicity, amount ingested, and animal/species susceptibility (Fowler, 1981).

### Plants

The following factors of plants are linked to toxicity:

- The concentration of active ingredient varies among different species of plants.
- The entire plant can be toxic, but in some cases, parts of the plant are responsible for the toxicity (e.g., grain, leaves, and fruit).
- The active ingredient content can vary depending on the state of plant development; for example, *G. officinalis* becomes toxic only after flowering.
- Preservation procedures can destroy the active ingredients; for example, drying can reduce toxicity (e.g., *Cicuta* spp.).
- Herbicide treatments may increase the toxic principle content and can make animals crave plants more.

## POISONOUS PLANTS AFFECTING ANIMALS

### *Aconitum napellus* (Ranunculaceae family)

**Common names:** aconite, monkshood, and wolfsbane

*Aconitum napellus* has blue or white flowers bilaterally symmetrical with a prominent upper hood, which gives the genus its name. The perennial herb has palmate

leaves and a tuberous root. Several species are common garden plants, but cases of poisoning in European countries are not common. However, it should be remembered that these plants are potentially poisonous.

#### *Toxicity*

All parts of the plant contain toxic alkaloid aconitine, with the highest concentration in the tubers (roots) and seeds and lower amounts in the leaves and stems, but the alkaloid content and composition vary throughout the year. The alkaloid content is highest when plants are flowering (June and July). Horses, donkeys, and goats are more sensitive to aconitum than are sheep. The plant is not usually eaten (acrid test), and field poisoning is uncommon. Aconitine may also be present when forage or hay is contaminated by it.

#### *Clinical presentation*

The clinical signs produced by this plant include vomiting, colic, bradypnea and dyspnea, muscular weakness, paralysis, pupillary dilatation (mydriasis), and death. Death is due to asphyxia, and postmortem findings are those associated with suffocation. The lesions are not specific, usually appearing to be gastric and renal congestion (Lorgue *et al.*, 1996).

### ***Amianthium muscaetoxicum* (Liliaceae family)**

**Common names:** fly poison, stagger grass, and crow poison

This plant is found in open woods, fencerows, old fields, roadsides, and pinelands, particularly in clay soils, although it can also be found in sandy soils. These plants are perennial, subscapose, and bulbous herb. Leaves are mostly basal and linear. Flowers are white, turning greenish with age; they are borne in dense racemes. Plants are found mostly in moist, wooded slopes, meadows, open fields, and bogs.

#### *Toxicity*

The highest concentration of the toxin, an alkaloid, is found in the bulb of the plant. The alkaloid is also found in the fruit and leaves. Animals consume the plant only when other forage is unavailable. Cases of toxicity occur in the spring, summer, and fall. Cattle and sheep are most commonly affected.

#### *Clinical presentation*

Animals exhibit vomiting, frothing at the mouth, salivation, staggering, dyspnea, subnormal temperature, and weakness. Death is caused by respiratory failure.

### ***Cannabis sativa* (Cannabinaceae family)**

**Common names:** marijuana, marihuana, bhang, hashish, Ganja, and sinsemilla

The term marijuana refers to the dried leaves and flowers of the Indian hemp plant, which is cultivated illicitly for its psychological effects. It is an illicit drug plant, and its leaves, flowers, and seeds contain several cannabinoids that are favored by some people for their psychoactive properties. Originally, the plant *Cannabis sativa* was an annual herb that was native to Asia, but it has spread throughout the world. Marijuana or hemp has been employed for thousands of years as a source of fiber to make rope in Asia and the Middle East and as a medicinal and recreational drug. Only since the 1960s and 1970s has the recreational use of marijuana become common in the Eastern world. It is currently sold in the illicit drug trade and can be found growing in European countries – in out-of-the-way places, in flower beds, or in pots within homes or vacant lots. Marijuana is the term used to describe the dried leaves and flowering heads of the cannabis plant.

#### *Active principle*

The main psychoactive ingredient in the plant is the complex chemical delta-9-tetrahydrocannabinol (THC). This accounts for approximately 3 or 4% of the dry weight of the herbal material, although modern strains of the plant grown indoors under intensive cultivation conditions may contain as much as 15–20%. The pharmacological effect of this agent is to produce central nervous system (CNS) depression and derangement. THC is absorbed when taken by mouth, having a low oral bioavailability resulting from a large first-pass effect because of extensive liver metabolism (taking as long as 3 or 4 h to reach peak blood levels).

#### *Conditions of poisoning*

The quantity of the active ingredient exposed varies markedly according to the variety of the plant, the sex of the plant, the geographical location, the stage of growth, and the nature of the growing season. The greatest concentration of the active ingredient is in the flowering tops of the female plant, whereas leaves are less potent and seeds contain little of the active ingredient.

Individuals involved in the use of illicit drugs frequently keep pets that have access to the plants, or to products made from the plants, and that can potentially cause marijuana poisoning. Some individuals delight in involving their pets in the drug experience with them. In other cases, accidental ingestion results in toxicity to the pet.

### *Clinical presentation*

Clinical signs seen in humans at high doses of marijuana include hallucinations and strange fantasies, and the user can no longer hold a coherent conversation. There is commonly a sudden stimulation of appetite, particularly for sweet foods. These effects may be followed by tiredness and sleep.

Animals show behavioral abnormalities and hyperexcitability. The most affected animal is the dog. The main clinical signs in the poisoned dog are vomiting, salivation, incoordination, alternating somnolence and hyperactivity, muscular weakness, and hyperthermia. Moreover, CNS and respiratory depression is another important sign in the dog. This is followed by coma and possibly death.

### ***Colchicum autumnale* L. (Liliaceae family)**

**Common names:** *colchium, meadow saffron, autumn crocus, and naked ladies*

*Colchicum autumnale* is locally abundant in meadows.

### *Toxicity*

All parts of the plant *C. autumnale* contain the alkaloids colchicine and colchicine, of which the former is more toxic. Colchicine exists in all parts of the plant, but its highest concentration is in the bulb (~0.8% by weight). The pale purple flowers contain approximately 0.1% colchicine by weight. Both alkaloids withstand storage, drying (persist in contaminated hay), and boiling. At doses of 0.25 mg/kg body weight (BW), colchicine has a purgative effect. At higher doses, colchicine is a potent gastrointestinal toxin and causes intractable multiorgan failure. The European native is located in wet meadows and with poisoning occurring during the spring. Poisoning of animals in the spring involves ingestion of the young leaves, whereas in the autumn the flowers of plants growing wild in pastures are implicated (Humphreys, 1988).

### *Clinical presentation*

Poisoning primarily affects cattle but can affect equines and swines raised on pasture. Clinical signs appear approximately 48 h after ingestion. In cattle, the clinical signs of intoxication are predominantly related to the digestive tract and are characterized by salivation, dysphagia, colic, abdominal pain, diarrhea, and fetid feces that are green or black with tenesmus. Death occurs from cardiorespiratory failure and may be delayed for several days depending of the amount of plant ingested. The visible postmortem finding is gastroenteritis. Lesions

that appear are edema and intestinal bleeding. In the horse, abdominal and thoracic serous effusions also occur.

### ***Conium maculatum* (Umbellifereae family)**

**Common name:** *hemlock*

This is a glabrous, branching biennial herbaceous plant, with hollow purple-spotted stems arising from a thick taproot. The poison hemlock usually has only one fleshy taproot, there are no pithy partitions in a hollow area at the juncture of stem and root, and stem and upper stem leaves are divided. Leaves resemble parsley, and they have a parsnip odor when crushed. This is probably more common than spotted water hemlock and found in drier, more upland habitats. It is found in roadside ditches and in damp waste areas.

### *Toxicity*

The poison hemlock contains piperidine alkaloids (coniine and cyanapine) and other compounds that are capable of poisoning livestock, poultry, and humans. The stems, leaves, and mature fruits are toxic. The leaves are more dangerous in spring, and the fruit is most dangerous in fall.

### *Clinical presentation*

Symptoms are gastrointestinal irritation and neurological effects (nervousness, trembling, staggering, and coldness of the extremities), hyperpnea, and tachycardia (Lorgue *et al.*, 1996). Eventually, coma and death occur from respiratory failure. In general, animals can recover within hours.

### ***Convallaria majalis* (Liliaceae family)**

**Common names:** *lily of the valley, may lily, Jacob's lily, and our lady's tears*

This is a native European perennial plant of garden or wood that contains a poisonous substance in the roots and stems. The leaves are long and broad, typical of the Liliaceae family. It is an ornamental and houseplant. The animals most affected are dogs, cats, and poultry. Dogs and cats usually chew and/or swallow the flowers, and birds (poultry, duck, and geese) usually ingest the fresh plants.

### *Toxicity*

The plant contains cardiotoxicants and convallatoxins.



### *Clinical presentation*

Poisoned animals are often found dead. Occasionally, terminal seizures are evidenced. Symptoms vary from mild gastrointestinal perturbation (vomiting and diarrhea) to terminal cardiac dysfunction (arrhythmias and bradycardia).

## ***Coriaria myrtifolia* (Coriariaceae family)**

**Common names:** tanner's herb, Mediterranean coriaria, and redoul

This is an ornamental plant that grows in the Mediterranean countries of Europe.

### **Toxicity**

*Coriaria myrtifolia* contains coriamyrtine, which can cause convulsions similar to those produced by strychnine or picrotoxin. The entire plant is toxic, and the poisoning occurs by ingestion of the stems, branches, and fruits.

### ***Clinical presentation***

The frequency of poisoning is high in goats and low in cattle and horses. Goats of all ages are very sensitive in regions in which redoul (imported breeds) are present. The number of affected individuals in herds varies from 10 to 50%. The lag time is short, 20 min (sometimes <5 min) to 2 h after ingestion of the plant (Faliu *et al.*, 1985).

Mild symptoms are characterized by drunkenness, ptialism, mydriasis, sometimes chills, loss of appetite, and bloating. These symptoms are followed by a severe form characterized by nervous signs such as retching, trembling, violent seizures that last 10–15 min, muscular contractions, ataxia, anorexia, tachycardia, intense tachypnea and dyspnea, nystagmus, mydriasis, ptialism, and bloating. Death can occur within 20 min to 2 h after a crisis by respiratory arrest (20–50% of cases), or there may be a progressive cure. The lesions are not specific and are characterized by severe and generalized congestion, edema and cyanosis of the mucous membrane (Faliu *et al.*, 1985; Lorgue *et al.*, 1996).

## ***Datura* spp. (Solanaceae family)**

**Common name:** thorn apple

All species of *Datura* are poisonous. The following species may be found where pets may be exposed:

- *Datura stramonium* (jimsonweed or Jamestown weed)
- *Datura metaloides* (thorn apple, apple of Peru, and tolguacha)
- *Datura arborea* (trumpet vine and angel's trumpet)

These species and others are grown as ornamentals, such as trumpet vine, or occur as weeds. The plants vary in appearance, but all have large, tubular flowers ranging in color from white to lavender. The fruit is an ovoid spiny capsule, giving rise to the common name thorn apple. Most of the plants emit an objectionable odor.

### *Toxicity*

Poisonous substances found in *Datura* are tropane alkaloids (atropine, hyoscyne, and scopolamine) and hyoscyamine in all parts of the plant, particularly in the seeds. It is found in hay or silage. The foliage may contain as much as 0.25–0.7% alkaloids. The seeds are likely to be the source of toxicity for pets.

### *Conditions of poisoning*

In the illicit drug culture, *Datura* seeds are circulated to produce hallucinations. As with other plants involved in the illicit drug trade, pets are likely to consume toxic material accidentally if their owners are careless in handling the plant. Occasionally, a person may deliberately give the material to his or her animals.

### *Clinical presentation*

Clinical signs associated with *Datura* poisoning are similar to those caused by an overdose of atropine. The signs may occur a few moments after ingestion or may not appear for several hours. The poisoning has an acute course in which there is weak, rapid pulse and intense heartbeat; disturbances of vision (dilated pupils due to mydriatic effects of *Datura*); dry mouth; incoordination; convulsions; and coma.

## ***Hypericum perforatum* (Hypericaceae family)**

**Common names:** St. John's wort, goatweed, and Klamath weed

There are various species of *Hypericum*, but the only species of importance in Europe is *Hypericum perforatum*. This perennial plant has a habitat and distribution in dry soil, wood, pastures, ranges, and along roadsides.

### *Toxicity*

The photodynamic pigment (hypericin), a red fluorescent found in the black dots that are scattered over the surface of the leaves, stems, and petals, can be absorbed and is capable of sensitizing the pigment of animals to sunlight.

### *Clinical presentation*

The clinical signs occur after 48 h to 21 days of grazing the plant or as a result of ingesting contaminated hay

or fodder. This delay depends on the time required for hypericin to reach a critical concentration in the skin and also the intensity and duration of sunlight to which animals are exposed (Faliu *et al.*, 1986).

The animals most affected are cattle, horses, sheep, and goats; pigs are rarely affected. The poisoning occurs exclusively when the animals are exposed to sunlight and is characterized by photosensitization, severe pruritus (head, ears, face, and extremities) and dermatitis with ulceration, erythema, blindness, convulsions, diarrhea, and extreme hypersensitivity to touch and to contact with cold water. The lesions observed are mainly cutaneous (dermatitis), degeneration of liver, and inflammation of the gallbladder. For example, in cattle, the affected skin peels off in patches resembling leather. Deaths have been described. In horses, the white areas or mucous membranes are the site of depigmented erythema pruritis, followed by edema (Faliu *et al.*, 1986).

### ***Galega officinalis* L. (Papilionaceae family)**

**Common name:** goat's rue

*Galega officinalis* is a perennial European legume with a lush alfalfa-like appearance in early growth, but it becomes very rank when mature. It is a perennial herbaceous plant with a hollow stem and elliptical or lanceolate leaves. The flowers are light blue to violet in color. The plant is unpalatable to livestock, particularly when mature, and its use as forage was advocated only for very young plants.

#### *Toxicity*

This leguminous plant contains two nitrogen guanine components, galegin (isoamylene guanidine) and hydroxygalegin, that are present in all parts during flowering and fruit formation. These substances have pharmacological properties as hypoglycemic and galactogenic agents. Galegin decreases blood pressure and paralyzes the CNS. This plant appears in summer, especially during the months of July and August when the plant is in flower or producing pods, and it is found in the southern area of France. Accidental poisoning affects mainly sheep, but some cases have also been seen in cattle (Bezard *et al.*, 2002). *Galega officinalis* L. is generally rejected by cattle, probably because of the plant's nasty smell and bitterness, especially when freshly cut. In case reports describing cattle and sheep fed the same hay, only sheep ate the toxic plant and were poisoned. The poisoning is usually related to feeding dry hay or freshly cut fodder contaminated by *Galega*, and it is assumed that fodder containing no more than 10% *Galega* will be toxic for sheep (Puyt *et al.*, 1981).

#### *Clinical presentation*

The latency period usually lasts 12–24 h after ingestion of the plant. It is characterized by an asphyxic syndrome caused by hydrothorax, which leads to death within hours. A voluminous hydrothorax is observed during postmortem examination.

### ***Nerium oleander* (Apocynaceae family)**

**Common name:** oleander

*Oleander* is a very common ornamental plant in Mediterranean areas of Europe; it is also grown in temperate climates in conservatories and greenhouses. *Oleander* is a large ornamental evergreen shrub. The long, pointed leaves are oblong and contain a prominent midrib. Large clusters of white, pink, or red flowers appear at the end of the branches in summer. It is particularly abundant on sandy soils near the coast. It is widely cultivated and used as landscaping along roadsides and edges of woods and in lawns and gardens.

#### *Toxicity*

Oleander has been a well-known poisonous plant since classical times, with all parts containing very toxic cardiac glycosides (oleandrin, digitoxigenin, neriin, folinerin, and rosagenin) that apparently exert a digitoxin-like effect (cardiotoxic potential) (Langford and Boor, 1996). Toxins may also be inhaled in smoke when plants are burned. Human poisoning occasionally occurs from eating hot dogs roasted on sticks from nearby oleander plants. This extremely toxic plant can poison livestock and humans at any time of the year. Lethal doses in horses, donkeys, and calves were reported to be 30–50 mg/kg BW (Oryan *et al.*, 1996).

#### *Clinical presentation*

Severe gastroenteritis, diarrhea, abdominal pain, sweating, and weakness are the usual symptoms. These signs appear within a few hours after eating the leaves. Cardiac irregularities are common, often characterized by increased heart rate. However, a slower heart rate is often detected in the later stages. In comparison with other species, turkey poults have not been found to be very sensitive to oleander.

### ***Pteridium aquilinum* L. or *Pteris aquilina* L. (Polypodiaceae family)**

**Common name:** Bracken fern

*Pteridium aquilinum* is also classified by some authorities as three separate species: *P. aquilinum*, *P. esculentum*, and *P. yarrabense*.

It is distributed throughout all European countries and is most common in woods, old fields, waste places, and roadsides, particularly on relatively dry sites. The poisoning is common in places where *P. aquilinum* grows naturally.

### Toxicity

In the poisoning by *P. aquilinum*, a polioencephalomalacia occurs because these plants contain the enzyme thiaminase, which inactivates thiamine (vitamin B<sub>1</sub>) in cattle, sheep, and horses; this is called "pteridism." The thiaminase hydrolyses vitamin B<sub>1</sub>, leading to a depletion of vitamin B<sub>1</sub> and producing a clinical syndrome of thiamine deficiency primarily in horses.

Ingestion of the green plant, during periods of food shortage or when dried plant material (dryness does not reduce toxicity) is mixed in with hay or straw, over a prolonged period (15–30 days) is toxic. Toxic effects by the plant appear to be cumulative, and symptoms may not appear until several weeks or months later (1–3 months), depending on the species of the animal, the amount ingested, the time of year, and other factors. Clinical cases are most often seen in the spring or late summer or fall, especially after periods of drought when other forage is short or not available. Animals have shown toxicity from consuming hay containing the dried plants.

In ruminants, an aplastic anemia factor causes depression of the bone marrow; this effect is cumulative but appears over a short period of time. Sheep are less susceptible than cattle and horses to the toxic effects.

### Clinical presentation

It causes poisoning in two unique ways. First, ingestion by ruminants over a short period causes depression of bone marrow activity leading to pancytopenia manifested mainly as ecchymotic hemorrhages in mucosae, septicemia, and severe diarrhea. Second, ingestion over a long period causes proliferative lesions in and bleeding from the urinary bladder mucosa.

Cattle may exhibit two types of symptoms. The laryngeal form is often seen in younger animals and is characterized by edema of the throat region, resulting in difficult and loud breathing. The enteric form may be preceded by the laryngeal form and is characterized by bloody feces and urine and excessive bleeding from fly bites. The blood is slow to clot because platelets are deficient. Death usually occurs within a few days after symptoms appear. Sheep have shown blindness due to progressive retinal atrophy after grazing bracken fern; affected animals are permanently blind and adopt a characteristic alert attitude.

Horses exhibit muscular weakness and tremor, motor incoordination, and a crouching stance with arched neck

and feet placed wide apart. Occasionally, a fever up to 40°C is present. Before death, horses may "head press" objects and have spasms with the head and neck drawn backwards. Death is preceded by tonic-clonic spasms and opisthotonus. The disease is almost always fatal, and the lesions in cattle are indicative of a general hemorrhagic syndrome. Necropsy reveals multiple hemorrhages throughout the carcass; necrotic ulcers may be present in the gastrointestinal tract (abomasum and intestine).

In differential diagnosis, other plants, such as *Equisetum arvense* (horsetail) and *Beta vulgaris* (turnip), should be taken into account because these plants also induce thiamine deficiency. In horses, the condition must be distinguished from other neurological disorders produced by poisonous plants, such as *Crotalaria* spp. or *Senecio jacobaea* (ragwort).

## *Quercus* spp. (Fagaceae family)

**Common names:** live oaks (evergreens) and acorns

Different species of oak are responsible for poisoning. Acorns are the fruits of different species of oak, which have a detachable cap covering a hard oval body. Common in England and northern Europe are *Quercus ilex* (holm oak or evergreen oak), *Quercus pedunculata* (pedunculate oak), *Quercus robur* (common oak or English oak), and *Quercus petraea* syn. *sessiliflora* (sessile oak or durmast oak).

### Toxicity

Most animals are susceptible, although cattle and sheep are most often affected. Acorns and oak leaves contain large amounts of tannic acid along with small amounts of a volatile oil. The toxic principle, which appears to be gallotannins or their metabolites, causes gastrointestinal toxicity and renal damage. The incidence of poisoning is often associated with weather conditions; a hot dry summer and an abundant crop of acorns in the fall when grass is rare and also winds cause acorns to fall while still green. Pigs are resistant to pyrogallol and tannic acids (Lorgue *et al.*, 1996). Tannic acid is used as an astringent.

### Clinical presentation

Consumption of large quantities of young oak leaves in the spring or green acorns in the fall produces clinical signs approximately 4 weeks after ingestion. The toxic syndrome is characterized by gastrointestinal and renal dysfunction (Barbier, 2005).

Green and unripe acorns are more toxic than brown acorns and contain the highest levels of pyrogallol acids (up to 8%). In addition, certain species of oak (e.g., the pedunculate oak) are more toxic than others. The age of the tree is significant, with younger trees having the

highest levels of pyrogalllic acids and tannins (Lorgue *et al.*, 1996). The tannin content decreases with maturity. Cattle are most affected, especially young animals (1–3 years); sheep are not sensitive, and goats are rarely affected.

It is considered that the high tannic acid content is responsible for the toxicity. Tannins have a direct action by decreasing digestive secretions and have a constipating effect as an indirect action after hydrolysis in the rumen. The metabolites gallic acid and pyrogallol are water soluble, and they are well absorbed in the gut. The metabolites are responsible for liver and kidney damage as well as necrotic lesions in the intestine.

Clinical signs in cattle include anorexia and prostration often accompanied by low temperature, depression, emaciation, rumen stasis, serous nasal discharge, polydipsia, polyuria, and constipation followed by mucoid to hemorrhagic diarrhea, dark fetid diarrhea, and sometimes blood clots. Nephrosis is another sign of toxicity. In sheep, the clinical signs are characterized by somnolence, leading to staggering and going down (Humphreys, 1988). In horses, the signs observed include dullness, hypothermia, weakness, inability to walk, incoordination, mouth ulcers, mucosa discoloration, patchy sweating but no colic, passage of hard or sloppy feces, and red or brown urine (Warren and Vaughan, 1985). The rumen contains acorns in large numbers. Evolution is frequently fatal. Hemorrhagic lesions of the gastrointestinal tract, nephritis, and perirenal edema may be important.

### ***Ricinus communis* (Euphorbiaceae family)**

**Common name:** *castor bean*

This plant has large, palmately lobed leaves, and it is a robust annual (in southern regions) or perennial (in tropical and subtropical regions) woody herb. It is cultivated and occasionally escapes and persists in pinelands, waste places, and roadsides.

Flowers are green and inconspicuous; staminate flowers are near the base, and pistillate flowers are mostly near the top of a small panicle. The fruit is a three-lobed capsule with a soft, spiny exterior, 1.5–2 cm long. There are three seeds per capsule. Seeds are shiny, grayish brown mottled with reddish brown, and 10 mm long and 6 or 7 mm wide. Seeds resemble engorged ticks and usually tree in a somewhat spiny pod.

#### *Toxicity*

The poisonous principle is a phytotoxin called ricin. In Europe, the plant is commonly planted not only as an ornamental but also in vegetable gardens to repel moles. Horses are most susceptible to poisoning, but all

livestock and humans can be affected. All parts of the plant are toxic, especially the seeds. Toxicity is seen most often in spring and summer.

#### *Clinical presentation*

Animals are most often poisoned when feed grains have become contaminated with the castor bean seeds. Depending on the amount consumed, symptoms appear from several hours to days after animals consume the toxin. Violent purgation in the form of straining and bloody diarrhea is the classical sign. Other signs are dullness, abdominal pain, weakness, trembling, and incoordination.

### ***Robinia pseudoacacia* (Papilionaceae family)**

**Common names:** *false acacia, locust tree, and black locust tree*

This is a large ornamental tree with alternate, compound leaves and unbranched spines resembling rose thorns. This plant has been cultivated and is a common tree in dry woodlands, fencerows, old fields, roadsides, and pinelands, particularly in clay soils, although it can be found in sandy soils.

#### *Toxicity*

Toxic principles include the phytotoxin robinin and the glycoside robitin (emetic and purgative) found throughout the plant, although the flowers have been suggested to contain the toxic principles. Apparently, the toxin is heat labile, and it produces gastrointestinal distress. Horses, cattle, sheep, goats, poultry, and humans may be poisoned by ingesting roots, bark, sprouts, seed pods, or trimmings during periods of drought or food shortage. Horses are most susceptible to the effects of *R. pseudoacacia*.

#### *Clinical presentation*

Symptoms include diarrhea, anorexia, weakness, posterior paralysis, depression, mydriasis, loss of appetite, irregular pulse, and difficulty breathing. Death is not frequent. Postmortem lesions are restricted to the gastrointestinal tract.

### ***Senecio* spp. (Compositae family)**

**Common names:** *ragwort and groundsel*

The genus of this family contains more than 1200 species, of which 25 are known to be toxic and cause seneciosis. Members of the plant family include *S. aquaticus* (marsh ragwort), *S. jacobea* (ragwort or tansy ragwort),



*S. squalidus* (Oxford ragwort), and *S. vulgaris* (groundsel). In the United Kingdom, there have been many reported incidents involving exposure to ragwort.

#### Toxicity

These plants contain pyrrolizidine alkaloids including jacobine, jacidine, jaconine, and retrorsine, which are all hepatotoxic. Retrorsine is the most widely distributed pyrrolizidine alkaloid. Most of the *Senecio* species are also found in *Crotalaria* spp.

#### Clinical presentation

Seneciosis is an acute and chronic hepatic insufficiency caused by ingestion of plants containing senecio. Poisoning occurs when the fresh plant is ingested (in pastures) or when it contaminates hay or silage (Vos *et al.*, 2002). All animals can be affected, but cattle and horses are especially affected.

Poisoning can present after a latent period from weeks to months. The clinical picture is characterized by a syndrome of photosensitization, jaundice, and CNS derangement. Clinical signs are extremely diverse and include anorexia, weight loss, constipation, mild jaundice of the mucosae, reduction in vision followed by blindness, ataxia with trembling, weakness, and drowsiness. Generally, poisoning results in a fatal outcome. The lesions are characterized by enlargement and congestion of the liver, hepatitis (a cirrhotic form) with ascites, nephritis, and edema of the abomasum (Lorgue *et al.*, 1996).

Most outbreaks occur in animals on pasture, but housed animals feeding on hay contaminated by the foliage or seeds may also be poisoned. In horses, photosensitization may develop following plant ingestion. Pyrrolizidine alkaloids can be passed into the milk of lactating animals (Goeger *et al.*, 1982).

### *Taxus baccata* L. (Taxaceae family)

#### Common name: yew

The yew (*Taxus baccata*) has been noted as the most poisonous plant in all of Europe. The yew is a tree or shrub of the family Taxaceae that will accommodate all types of soils and grows in shade and sun. It is planted in parks, gardens, and cemeteries, especially for ornamental fences. Other yew species are also toxic; *T. cuspidata* (Japanese yew) and *T. canadensis* (Canada yew) are widely planted in gardens. Poisoning occurs in animals located in pastures, woods, or gardens or as a result of careless disposal of yew clippings or leaves. All parts of the tree are poisonous, and all species of animals are susceptible. The leaves are harmful at all stages of plant growth, and drying and

storage do not lessen their toxicity. The sexes are separate, and the pistillate plants bear bright scarlet fruit in the fall. The red flesh covers a hard seed, and although the flesh is not poisonous, the seed is extremely poisonous. The sap of the tree contains volatile oils that are irritants, and the leaves contain alkaloids taxines (taxine A and taxine B) and ephedrine and cyanide (hydrocyanic acid). The taxine is found in all parts of the tree except the fleshy red part of the berry. These compounds are capable of causing hypotension, bradycardia, and depressed myocardial contractility and conduction delay, similar to digitalis poisoning (Alexander *et al.*, 1946).

#### Toxicity

Chewing seeds has proven fatal to animals and humans. The foliage is even more toxic, and it is this that proves so poisonous to cattle, sheep, goats, pigs, and horses. There are probably more problems with children eating these berries than any other, except possibly pokeweed. Chewing these seeds and then swallowing can cause serious poisoning. The relatively greater resistance of ruminants is probably due to the dilution of the ingested material by the rumen contents. This effect may also account for the individual variation in susceptibility of different animals and for the delay, sometimes seen in cattle, before clinical signs of yew poisoning become apparent (Humphreys, 1988).

#### Clinical presentation

The conditions of poisoning are by consumption of leafy branches or lying *in situ* (fall) in the soil after harvest or a thunderstorm. The most common feature of yew poisoning is sudden death (1–48 h depending on the amount ingested). The clinical signs observed are nervousness, trembling, ataxia, dyspnea, and collapse (Barbier, 2005). Bradycardia progresses to cardiac standstill, and death ensues without struggle. Death occurs within approximately 5 min. Empty right heart, dark tarry blood in left heart, and limited nonspecific postmortem lesions such as congestion of the kidneys, liver, spleen, and lungs are seen (Ogden, 1988). The presence of yew leaves in the rumen contents may help in making the diagnosis of yew poisoning.

### *Thuja occidentalis* L. (Cupressaceae family)

#### Common name: thuja

Thuja shrubs are found throughout Europe. They are frequently planted in parks and gardens for ornamental purposes, especially in enclosures. Animal species commonly affected are cattle, sheep, and, rarely, goats. Poisonings occur by consumption of branches or

dropped branches dropped on the ground after being cut down or following a storm.

### *Clinical presentation*

Peracute intoxication occurs within a few minutes, especially in sheep. Clinical signs of acute or subacute intoxication are essentially related to the digestive tract (hypersalivation, colics, bloating, and bloody diarrhea) (Jean-Blain and Grisvard, 1973). Toxicity progressing to dyspnea and paralysis accompanied by a cooling of the extremities. Death can occur between 1 and 48h depending on the amount of plant parts ingested. The lesions are characterized by discrete congestive gastroenteritis, which is not specific. The presence of thuja leaves in the rumen contents confirms poisoning.

## TREATMENT

Management of poisoning by toxic plants is complicated by the lack of antidotes for most plant toxins. The effect of treatment may be difficult to determine because of the wide variability in toxic reactions to the same plant. Table 78.3 describes some of the systemic effects of poisonous plants.

### Nonspecific treatment

The most important aspect of emergency medicine treatment is to ensure adequate physiological functioning. Most plant poisonings respond to supportive care. Frequently, the veterinarian must treat the animal before plant identification is done. The recognition of certain syndromes characteristic of specific plants is of importance.

Many plant poisonings will not be diagnosed at the onset of the disease. Therapy will, of necessity, be symptomatic. Even when a positive diagnosis can be made, there are rarely any specific antidotes. The most important step

in therapy for any plant poisoning case is to remove any residual plant materials from the digestive tract as quickly as possible.

Not all plant poisons have antidotes to reverse the toxic effects, but a few general principles might get one through the initial phase of management. Removal of unabsorbed poison from the gut can be achieved by several means, including induction of emesis, gastric lavage, and use of activated charcoal, tannins, and cathartics. Tannins and activated charcoal may be administered in a water slurry via oral route.

In emergency medicine and for critical patients, we typically start resuscitation with the airway and then breathing and circulation in an overdose scenario. The therapeutic management strategies can be summarized as follows: establishment of a patent airway, artificial respiration, cardiac massage, and perhaps the application of defibrillation techniques. Following stabilization of vital signs, the clinician may proceed with subsequent therapeutic measures. In plant poisonings, there is neither a specific treatment nor an antidote. The treatment involves enhancing the removal of the absorbed poison and providing supportive therapy to combat the effects of the poison already absorbed.

### *Decontamination procedures*

Ingestion is the most common route of poisoning in large and small animals. Decisions about gastric emptying depend on an accurate identification of the plant responsible for the poisoning because the majority of casual ingestions require no specific therapy. Once identified, decontamination is based on plant toxicity, symptoms, and time since ingestion. Unless contraindicated, emesis is preferred to lavage because plant pieces are difficult to remove by gastric tube in some animal species, particularly those that are small.

#### *Gastric decontamination*

Vomiting can be induced in the conscious patient by the administration of apomorphine (0.04mg/kg intravenously (i.v.) or 0.08mg/kg intramuscularly (i.m.) or subcutaneously). Apomorphine may cause respiratory and CNS depression and may induce protracted emesis, ultimately resulting in a severely debilitated animal. These effects can be controlled by using a narcotic antagonist such as naloxone (0.04mg/kg i.v.).

There are two decontamination procedures – gastric lavage and irrigation. When it is known that an animal has ingested a potentially poisonous substance within less than 2h, efforts should be made to remove it from the upper gastrointestinal tract. If the animal has not vomited and can swallow, an emetic should be given promptly. In the case of an irritating toxin, rinsing of the mouth with sodium gluconate for at least 2h can be performed.

TABLE 78.3 Systemic effects of poisoning plants

Gastrointestinal irritants	<i>Ricinus communis</i> , Araceae family
Cardiovascular abnormalities	<i>Convallaria majalis</i> , <i>Digitalis purpurea</i> , <i>Aconitum</i> spp.
Renal dysfunction	<i>Datura stramonium</i> , <i>Cicuta</i> spp.
Hepatic dysfunction	<i>Senecio</i> (pyrrolizidine alkaloids)
Convulsions	<i>Cicuta</i> spp.; <i>Coriaria myrtifolia</i> ; <i>Aconitum</i> , <i>Taxus</i> , and <i>Veratrum</i> plant species
Nicotine symptoms	<i>Conium maculatum</i>
Atropine symptoms	<i>Datura stramonium</i>

Gastric lavage is an important emergency procedure, but it is relatively ineffective in suspected poisoning cases in which significant time has elapsed. Changes in techniques (e.g., using a larger tube, more volume, and more frequent lavages) have proven this to be a useful procedure when undertaken within 2h of ingestion of a toxicant (Hanna, 1986). Gastric lavage is recommended if the animal is presented within 60min of a potentially lethal ingestion of a plant. Proper placement of the patient may help prevent aspiration and improve the yield of the procedure. If more than 2h has passed since ingestion of the plant material, it is likely that the stomach will have already emptied into the intestine. In such cases, subjecting the pet to further stress through induced emesis is unwise. The patient should be sedated and as large a gastric tube as possible should be inserted to facilitate aspiration of the ingesta back out through the tube.

The use of activated charcoal has revolutionized the treatment of poisoning. Due to its small particle size and enormous surface area, it can adsorb a large amount of toxins. The usual dose is 1 g/kg body weight or 10 parts of activated charcoal for every 1 part of toxin, whichever is greater (large animals, 250–550 g; small animals, 5–50 g). Activated charcoal will be effective for preventing absorption of toxicant when it is properly utilized. It can be administered after vomiting has occurred or can be used in the gastric lavage solution. Single-dose activated charcoal is more beneficial if given within 60min of a toxic plant ingestion. The “universal antidote” should be avoided because magnesium oxide and tannic acid interfere with the adsorption capacity of activated charcoal. Activated charcoal adsorbs most alkaloids well and should be administered in almost all symptomatic cases. Other adsorbents, such as magnesium oxide and kaolin, are less effective than activated charcoal.

Ipecac syrup and cathartics have not been shown to be of benefit in cases of ingestion of poisonous plants. However, the use of emetics is recommended if the animal is conscious and the absorption time is less than 2h. Apomorphine can be injected subcutaneously at a dose of 0.2 mg/kg, but it is effective only in dogs. Ipecac syrup up to 10% at a dose of 1 or 2 ml/kg can be used in cats and dogs. Xylazine is also an effective emetic, particularly in cats (0.05–1 mg/kg i.m.), but it is less effective in dogs.

Gastric lavage is practical and effective if ingestion of the poison is recent (<2h). Gastric lavage is performed with 5–10 ml/kg of isotonic sodium chloride and contains activated charcoal in suspension. After lavage, a laxative agent may be placed into the stomach to further evacuate the intestine. Mineral oil is a suitable laxative. The dosage for small animals is 5–30 ml in dogs and 2–6 ml in cats. An oral saline cathartic of sodium sulfate (0.5 g/kg) is an efficient agent for evacuation of

the bowel and may be used in an emergency in small animals. Magnesium sulfate (1 g/kg) and castor oil (10–60 g per animal) can also be used; either can be used instead of mineral oil if desired. A high colonic enema is indicated to evacuate the toxicants from the lower gastrointestinal tract. Warm water with castile soap makes an excellent enema solution. Other enema preparations can be useful. The entire digestive tract of a dog can be emptied by a judicious combination of a gastric lavage and a high colonic enema. Care should be taken to avoid the induction of dehydration and electrolyte and the acid–base imbalances once the gastrointestinal tract has been evacuated.

Gastric aspiration and serial activated charcoal are useful in nicotine and related poisonings because of the secretion of nicotine into the acid milieu of the stomach of carnivores.

#### *Enemas*

This is a technique very useful for removing toxins from the lower gastrointestinal tract. The best enema solution is lukewarm water that is infused into the rectum of a patient. The enema should be repeated after 2h.

#### *Laxatives and purgatives (cathartics)*

Laxatives promote the elimination of soft, formed feces, whereas purgatives produce a more fluid evacuation. The following substances may be used for these purposes:

- Lubricant and emollient laxatives (fecal softeners): mineral oil (liquid paraffin) (cattle, 250–500 ml; horses, 250–1000 ml; pigs, 25–300 ml; dogs, 5–30 ml; cats, 2–6 ml).
- Bulk laxatives: methylcellulose (dogs, 0.5–5 g, and cats, 0.5–1 g, per os); carboxymethylcellulose sodium, psyllium, or plantago seed (dogs, 3–10 g, and cats, 3 g, per os); agar; wheat bran.
- Osmotic cathartics (saline purgative) – these are not absorbed and retain water in the intestinal lumen: magnesium sulfate (cattle, 250–500 g; horses, 30–100 g; pigs, 25–125 g; dogs, 5–25 g; cats, 2–5 g), sodium sulfate (cattle, 500–750 g; horses, 250–375 g; pigs, 30–60 g; dogs, 5–25 g; cats, 2–5 g), sugar alcohols (mannitol and sorbitol), lactulose (dogs, 5–15 ml, t.i.d., per os).
- Irritant cathartics: In this context, purgatives can be included with diphenylmethane or anthraquinone.

#### *Enhanced removal and elimination*

This procedure includes pH manipulation of urine or blood or both and other adjunct therapeutic modalities that may be useful in toxic situations. Administration

of fluids and diuretics may enhance the glomerular filtration rate. Fluids such as saline and lactated Ringer's solution are administered to promote urine production.

Osmotic diuresis may be induced by infusion of mannitol 10% solution or hypertonic glucose from 10 to 30% at 20–40 ml/kg. The application of diuretics such as furosemide (3–5 mg/kg) i.m. or i.v. injection may enhance urine production.

Forced acid diuresis to eliminate weak bases can be done with ammonium chloride orally (20–40 g in large animals, 100–200 mg/kg in dogs, and 20 mg/kg in cats) to maintain a urine pH of 5.5–6.5, with arginine choride i.m. or i.v. (7–10 g in large animals and 100–200 mg/kg in small animals), or with ascorbic acid i.v. (40 mg/kg in all animal species).

Forced alkaline diuresis to eliminate weak acids can be done with sodium bicarbonate 1.4% by i.v. infusion to maintain a urine pH higher than 7 (2–4 ml/kg during 24 h in large animals or given by slow, continuous administration for best results in small animals) and with Ringer's lactated by i.v. infusion (5–10 ml/kg per hour in all species).

### Specific treatment

Specific treatment is possible only in rare cases. For instance, in the case of intoxication by *Pteridium aquilinum*, horses can be treated with thiamine intramuscularly at a dosage of 100–200 mg per day for 7–14 days, or the anticyanide treatment can be used with sodium thiosulfate (20% solution), 4 ml/kg BW, strictly intravenously, and sodium nitrite (10% solution), 2 ml/kg BW, strictly intravenously.

## SUPPORTIVE THERAPY

Because antidotes are available only for a few toxins, treatment of most cases of poisoning is largely supportive. The aim of supportive measures is to preserve vital organ function until the poison is eliminated from the body and the patient resumes normal physiological functions, including control of body temperature, maintenance of cardiopulmonary function and renal system, control of acid–base status, alleviation of pain, and control of CNS effects with proper care for coma, seizures, hypotension, arrhythmias, hypoxia, and acute renal failure. Body temperature should be monitored. Hypothermia can be controlled with a circulating hot water blanket and by keeping the animal in a warm and draft-free cage. Hyperthermia is treated with ice bags, cold water baths, and enemas. It is vitally important that

the animal's body temperature be constantly monitored to prevent overcorrection.

Respiratory support requires an adequate patent airway using a cuffed endotracheal tube in an unconscious animal or by performing a tracheostomy under local anesthesia. A respirator is of great value, or an anesthesia machine may be utilized with manual compression of the bag.

It is more appropriate to insert an endotracheal tube and provide positive pressure ventilation if needed. A patient that is depressed sufficiently to require respiratory assistance must be continuously monitored.

Be prepared to supply respiratory support. Although analeptic agents such as doxapram may provide immediate support, the effect is transient and cannot be relied on to sustain a pet free from poisoning.

Cardiovascular support requires the presence of adequate circulating blood volume, cardiac function, tissue perfusion, and acid–base balance. Blood volume and cardiac activity are of most immediate concern.

In the presence of hypovolemia due to loss of both cells and volume, whole blood is needed. A sufficient quantity of whole blood should be given to raise the packed volume to 75% of the animal's estimated normal level. Hypovolemia due to fluid loss alone can be treated with the administration of lactated Ringer's solution or plasma expanders.

Cardiac activity can be aided by the application of closed-chest cardiac massage for immediate cardiovascular support, but administration of calcium gluconate, infused slowly intravenously, can stimulate cardiac activity in most instances. This agent is also a good non-specific treatment for many toxicities.

Control of acid–base balance problems is primarily a matter of physiologically maintaining an animal in a homeostatic condition. The most common acid–base disturbance seen is metabolic acidosis, but alkalosis may also occur in cases of poisoning. In correcting acidosis not of respiratory origin, sodium bicarbonate intravenously is the drug of choice. Caution must be exercised with alkalinizing agents to avoid the induction of alkalosis. For initial therapy, it is usually sufficient to administer 0.9% NaCl (physiologic saline) intravenously, followed by oral administration of divided doses of ammonium chloride.

Control of pain with a minimal dose of morphine or meperidine is used in animals. Management of CNS disorders in cases of intoxication is complex. Therapy depends on the presence of CNS depression or hyperactivity. Either disorder can easily be reversed to the extreme by overzealous treatment. CNS depression can also be considered with respiratory depression because the management of the two conditions is very similar. Although the intravenous administration of analeptic agents, such



as doxapram, is reported to be efficacious, their actions are short-lived, and CNS depression returns if the animal is not monitored continuously. Bemegride is also occasionally used to stimulate the respiratory centers in the medulla. Analeptics can also induce convulsions.

Cases of CNS hyperactivity, including convulsions, can be managed by the administration of CNS depressants or tranquilizers. Pentobarbital sodium, phenobarbital, phenytoin, and primidone are the agents of choice for convulsions and hyperactivity; care must be taken, however, because a respiratory depressing dose may be required to alleviate all adverse signs. Inhalant anesthetics are excellent for long-term management of CNS hyperactivity. Centrally acting skeletal muscle relaxants and minor tranquilizers (methocarbamol, glyceryl guaiaacolate, and diazepam) are also used for convulsant intoxicants. The animal should be placed in a quiet, dark room to reduce excitation due to auditory or visual stimuli.

## CONCLUSIONS

Compared to North American and African flora, European flora has fewer dangerous species; however, the number of plant poisons is considerable. Plant identification, the diagnosis of plant poisoning, the conditions conducive to plant poisoning, and predictive and toxicity factors are relevant in the field of plant toxicology. In general, plants cause poisoning only when eaten and affect livestock and companion animals. Many wild plants, houseplants, or illicit drug plants grown in Europe contain chemical substances in sufficient quantities to cause toxic effects in animals. *Liliacea* spp. and *Araceae* spp. are the major plant families involved in poisoning of companion animals; most cases are related to accidental ingestion of ornamental plants than wild plants, particularly at certain times of the year. In addition to the previously mentioned poisonous plants for dogs, the following plant families are also implicated: Euphorbiaceae, Apocynaceae, Solanaceae, Ericaceae, and Amaryllidaceae. Both specific and nonspecific treatments, including decontamination procedures, used in animal poisoning by plants are considered essential in veterinary toxicology.

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# Poisonous plants of Australia and New Zealand

*Rhian Cope and Kathy Parton*

## INTRODUCTION

Due to space constraints, this chapter largely focuses on the indigenous flora of Australia and New Zealand that has been noted to produce significant livestock losses and some of the major problematic introduced plants. It is not possible to cover all of the poisonous plants in Australia and New Zealand that are known to have caused livestock losses in a single chapter, and the reader is referred to more authoritative references for this. To allow for reasonable coverage of the most important species, the information is presented in tabular form (Tables 79.1 and 79.2).

The climate of Australia is unique compared with those of the other continents, and this has a substantial impact upon plant poisonings in grazing livestock. Australia is easily the driest of all continents: most of Australia is desert or semi-arid, and 40% of the landmass is covered by sand dunes. Eighty percent of Australia's landmass has an average annual rainfall of less than 600mm, with 50% of the landmass having less than 300mm. Rainfall for much of the continent is highly variable, and droughts, which may last several years, are a regular feature over much of the agriculturally productive areas of Australia. Accordingly, many of the toxicologically problematic indigenous plants are well adapted to this climate: they are extremely drought resistant (or their seeds are resilient), and they are able to very rapidly grow during the short periods of relatively suitable conditions. In these circumstances, poisonous indigenous flora often outcompete introduced fodder plants or it emerges earlier than more suitable fodder plants and poisonings result. Other common circumstances are that the indigenous plants can often be the last remaining plants available to livestock and again poisoning

results or those indigenous plants are cut and fed to livestock during drought, or that under drought conditions, cuttings of native plants are used as survival food for livestock.

The long biogeographic isolation of the Zealandia continent and the islands of New Zealand has resulted in a unique variety of native flora. Inevitably, this has resulted in a number of unique toxicological problems.

## REDUCING LIVESTOCK LOSSES ASSOCIATED WITH POISONOUS PLANTS IN AUSTRALIA AND NEW ZEALAND

The key factors in reducing losses due to plant toxicoses are an awareness of the types of potentially toxic plants relevant to the geographical area of production, an understanding of both the plant and the animal factors that influence the risk of poisoning, and an understanding of what indigenous plants can and cannot be used as feed for livestock under drought conditions.

A common, important plant-related factor in Australia is the ability of indigenous plants in the semi-arid and arid areas of the country to outgrow and outcompete introduced fodder plants, particularly following the onset of good conditions following drought. Often in such circumstances, the new plant growth contains relatively high amounts of relevant toxins. This situation, combined with hungry stock or stock that are new to a particular geographic area or both, is a recipe for significant stock losses. This type of scenario has been classically associated with the rangeland northern cattle

TABLE 79.1 Summary of major Australian plants that are hazardous to livestock

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Paterson's curse poisoning	<i>Echium plantagineum</i> (Paterson's curse; salvation Jane) Winter growing annual; grows initially as a rosette; flowering stems are produced in early spring; seeds in late spring; dies after flowering; rosettes have green to light green hairy egg-shaped leaves up to 30 cm long; flowers are generally purple; 2–3 cm long and shaped like curved trumpets; a stout taproot with numerous lateral roots is present.	Hepatogenous pyrrolizidine alkaloid (PA) toxicosis	Pyrrolizidine alkaloids; plant often has a high copper:molybdenum ratio; all parts and stages of the plant are toxic.	Widespread; highly invasive; introduced; high economic losses in sheep and cattle; significant infestations in temperate, winter rainfall areas of VIC and NSW, as well as the southwestern parts of WA, southern QLD, and the eastern half of TAS.	Overgrazing, drought and fire increase the pasture dominance of the plant; PA content is highest when flowering; not particularly palatable; pasture dominance of the plant in combination with reduced availability of alternative forages increases the risk; pigs, poultry, and horses (monogastric animals) are regarded as highly susceptible, cattle moderately susceptible, and sheep and goats the least susceptible; merino sheep and wethers relatively resistant compared with British breeds and Merino/British breed crosses; large variability in individual animal susceptibility.	Acute, subacute or chronic megalocytic hepatopathy. Horses: characterized clinically food refusal; depression; irritability; ill-thrift; evidence of hepatic insufficiency (icterus, secondary photosensitization, repeated yawning, head pressing, aimless wandering, compulsive walking, dyspnea secondary to recurrent laryngeal nerve dysfunction, and other signs of hepatic encephalopathy). Pigs: rarely seen except in grazed animals. Cattle: general ill-thrift; chronic weight loss and/or failure to thrive; unpredictable bouts of aggression; evidence of persistent diarrhea with tenesmus and secondary photosensitization. Sheep: deaths directly related to Paterson's curse are uncommon; PAs cause decreased productive life span; most common clinical issue found in sheep is copper toxicity rather than PA toxicity per se; PAs do not enhance copper accumulation per se, but they modulate the disease; <i>E. plantagineum</i> often contains a high copper:molybdenum ratio, which can induce copper toxicity in sheep; no effective treatment.	Relative copper excess/relative molybdenum deficiency toxidrome ("copper toxicity") in sheep due to high copper:molybdenum ratio in the plant; increased risk of ammonia poisoning (particularly when using nonprotein nitrogen dietary sources in ruminants); increased risk of pregnancy toxemia in sheep.

Viper's bugloss poisoning	<i>E. vulgaris</i> Similar to <i>E. plantagineum</i> Perennial or biennial rather than annual; Unlike <i>E. plantagineum</i> ; flowers are not arranged in a violin neck.	Hepatogenous PA toxicosis	Pyrrolizidine alkaloids; all parts and stages of the plant are toxic.	Less common than <i>E. plantagineum</i> ; mostly confined to the temperate tablelands of southeastern Australia.	As per <i>E. plantagineum</i> .	As per <i>E. plantagineum</i> .	May influence relative copper excess/ relative molybdenum deficiency toxidrome ("copper toxicity") in sheep; increased risk of ammonia poisoning (particularly when using nonprotein nitrogen dietary sources in ruminants); increased risk of pregnancy toxemia in sheep.
Blue heliotrope poisoning	<i>Heliotropium amplexicaule</i> Summer growing perennial; many hairy, branched stems radiating from a woody rootstock with a sender taproot; highly aromatic; extends over 1–2 m in diameter; leaves are alternate, dull green, and tapered at both ends; flowers are bluish-purple with yellow centers and are arranged in dense clusters along one side of a coiled stalk that resembles a violin neck; drought resistant.	Hepatogenous PA toxicosis	Pyrrolizidine alkaloids; all parts and stages of the plant are toxic.	Widespread in southeastern QLD and northern NSW; scattered colonies can be found in central and southern NSW, near Adelaide, in the Flinders ranges, and around Victor Harbour in SA.	As per <i>E. plantagineum</i> .	As per <i>E. plantagineum</i> . Significant causes of loss of young cattle; not a source of high copper:molybdenum ratio fodder, but like most PA-containing plants, can influence copper toxicity in sheep.	May influence relative copper excess/ relative molybdenum deficiency toxidrome ("copper toxicity") in sheep; increased risk of ammonia poisoning (particularly when using nonprotein nitrogen dietary sources in ruminants); increased risk of pregnancy toxemia in sheep.
European heliotrope poisoning; European turnsole poisoning	<i>H. europaeum</i> Summer-growing annual; prostrate to ascending up to 60 cm high; well-developed root system; emits a foul odor when crushed; leaves 1.5–9 cm long, 0.8–3 cm wide; ovate; paler below; leaf stalk to 4 cm long; flowers are arranged in two rows on either side of a caterpillar-like cyme; flowers are white with yellow throats; drought resistant.	Hepatogenous PA toxicosis	Pyrrolizidine alkaloids; all parts and stages of the plant are toxic; PA content is generally lower than <i>H. amplexicaule</i> .	Major noxious weed of temperate Australia; common in overgrazed or fallow land; recorded in all states except NT and TAS; greatest concentration is west of the Great Dividing Range in NSW and Victoria.	As per <i>E. plantagineum</i> .	As per <i>E. plantagineum</i> . Significant cause of loss of young cattle; not a source of high copper:molybdenum ratio fodder, but like most PA-containing plants, can influence copper toxicity in sheep.	May influence relative copper excess/ relative molybdenum deficiency toxidrome ("copper toxicity") in sheep; increased risk of ammonia poisoning (particularly when using nonprotein nitrogen dietary sources in ruminants); increased risk of pregnancy toxemia in sheep.

(Continued)



TABLE 79.1 (Continued)

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Heliotrope poisoning	<i>Heliotropium ovalifolium</i> Native to Australia; ascending to spreading perennial or annual (in adverse conditions) herb; 0.15–0.8 m high; flowers are white.	Hepatogenous PA toxicosis	Pyrrolizidine alkaloids; all parts and stages of the plant are toxic.	Kimberley region of WA	As per <i>E. plantagineum</i> .	As per <i>E. plantagineum</i> . Primarily associated with disease in horses, but other species are likely to be susceptible.	As per <i>E. plantagineum</i> .
Tansy ragwort poisoning	<i>Senecio jacobaea</i>	Hepatogenous PA toxicosis	Pyrrolizidine alkaloids; all parts and stages of the plant are toxic.	Humid temperate regions with annual rainfall > 750 mm; generally occur on heavy soils of moderate fertility; commonly found in areas cleared for pasture but never on properly developed or on poorly managed and/or; degraded pastures.	As per <i>E. plantagineum</i> . Somewhat less important in Australia compared with Paterson's curse and heliotropes.	As per <i>E. plantagineum</i> .	May influence relative copper excess/relative molybdenum deficiency toxidrome ("copper toxicity") in sheep; increased risk of ammonia poisoning (particularly when using nonprotein nitrogen dietary sources in ruminants); increased risk of pregnancy toxemia in sheep.
Fireweed poisoning	<i>S. linearifolius</i>						
Groundsel poisoning	<i>S. lautus</i>						
Cotton fireweed poisoning	<i>S. madagascariensis</i>						
African daisy poisoning	<i>S. quadrientatus</i> <i>S. pterophorus</i>						
Fiddleneck poisoning	Daisy-like; flower heads are normally rayed; completely yellow; the heads are borne in branched clusters.						
Fiddleneck poisoning	<i>Amsinckia calycina</i> <i>A. lycopsoides</i> <i>A. intermedia</i> <i>A. menziesii</i> Short-lived autumn/winter perennials; 20–70 cm tall; grow as a rosette from which a flowering stem is produced; flowers are tube-shaped, yellow or orange, and are arranged along a curved flower spike (a fiddle neck).	Hepatogenous PA toxicosis	Pyrrolizidine alkaloids; all parts and stages of the plant are toxic.	All of NSW and VIC; southern QLD; southeastern SA	As per <i>E. plantagineum</i> . Plants are unpalatable and poisoning occurs mostly when there are few or no other food sources.	As per <i>E. plantagineum</i> .	As per <i>E. plantagineum</i> .

<p>Kimberley horse poison</p> <p>Kimberly horse disease</p> <p>Crotalism</p> <p>Walkabout disease</p>	<p><i>Crotalaria crispate</i></p> <p><i>C. ramosissima</i></p> <p><i>C. dissitiflora</i></p> <p><i>C. linifolia</i></p> <p><i>C. mitchellii</i></p> <p><i>C. spectabilis</i></p> <p><i>C. verrucosa</i></p> <p><i>C. pallida</i></p> <p><i>C. juncea</i></p> <p><i>C. montana</i></p> <p><i>C. novae-hollandiae</i></p> <p><i>C. brevis</i></p> <p><i>C. eremaea</i></p> <p><i>C. retusa</i></p> <p>Herbaceous plants and woody shrubs; commonly known as rattlepods due to the fact that the seeds become loose in the pod as they mature and rattle when the pod is shaken; flowers are typically yellow and have the classical legume “pea-like” flower form.</p>	<p>Hepatogenous PA toxicosis; monocrotaline-induced proliferative pulmonary vasculitis; pulmonary hypertension and pneumotoxicity</p>	<p>Pyrrolizidine alkaloids; monocrotaline</p>	<p>Most areas of Australia with the exception of TAS</p>	<p>Kimberley horse disease/ walkabout disease is a major cause of mortality in horses in northern Australia; in general, all Australian <i>Crotalaria</i> species regarded as toxic to horses until proven otherwise; the plants are not palatable; poisoning usually occurs when there is a shortage of feed, which causes horses to graze indiscriminately; poisoning may occur when toxic plants are accidentally incorporated with conserved fodder such as hay; horses are 30–40 times more susceptible to the disease than are sheep and goats.</p>	<p>Clinical signs in horses relate to PA hepatopathy/ hepatic encephalopathy and include dull and depressed; often stand with their heads held down; anorexia; weight loss; icterus; muscle tremors (especially of the head and neck); frequent yawning; head pressing; copper-colored or red urine; episodes of frenzy and violent, uncontrollable galloping; difficulty swallowing (stop eating halfway through a mouthful of hay or grass); may appear to be blind; may aimlessly wander (walking in circles or bumping into objects); drag their hind legs (hooves have worn tips). Disease is untreatable; occasionally, horses develop pulmonary toxicity following ingestion of some <i>Crotalaria</i> sp.; the introduced plant <i>C. spectabilis</i> is most noted for this effect; pigs tend to develop nephrosis rather than a hepatopathy; disease in cattle often resembles that of <i>E. plantagineum</i>, although pulmonary disease can occur if monocrotaline is present.</p>	<p>Death from misadventure; increased risk of ammonia poisoning (particularly when using nonprotein nitrogen dietary sources in ruminants); increased risk of pregnancy toxemia in sheep.</p>
<p>Yellow wood poisoning</p> <p>McKenzie River disease</p>	<p><i>Terminalia oblongata</i></p> <p>Bushy tree 12m high; branching close to the ground; bark is dark gray and furrowed; leaves alternate, pale green to yellow green, with rounded tips; deciduous; flowers are tiny; fruits have a wing on both sides.</p>	<p>Hepatogenous photosensitization; nephrosis</p>	<p>Hydrolyzable tannins</p>	<p>McKenzie River Basin of QLD</p>	<p>Used as drought fodder will keep the sheep alive, but produces chronic kidney disease; cattle should be kept away from fallen leaves in winter if possible.</p>	<p>Nervous symptoms in sheep; acute disease in cattle (McKenzie River disease): abdominal pain; photosensitization; dehydration; dark brown urine, depression, limb stiffness, tremor; icterus, collapse; nephrosis; chronic disease in cattle is characterized by liver disease, nephrosis, and long-term ill-thrift.</p>	
<p>Poison peach</p>	<p><i>Trema</i> sp.</p>	<p>Hepatotoxic</p>	<p>Unknown</p>	<p>Coastal and inland north and eastern Australia</p>	<p>Potentially poisonous at all times.</p>	<p>Liver damage (periacinar hepatocyte coagulation necrosis); most animals die; death is usually rapid.</p>	<p>Resembles yellow daisy poisoning.</p>

(Continued)

TABLE 79.1 (Continued)

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Yellow daisy poisoning	<i>Wedelia asperima</i> Erect herb; yellow daisy-like flowers.		Kaurene (diterpenoid glycoside)	Northern Australian grasslands		Liver damage (periacinar hepatocyte coagulation necrosis)	Resembles poison peach.
Swainsona poisoning Darling or swainson pea poisoning	<i>Swainsona galegifolia</i> <i>S. canescens</i> <i>S. luteola</i> <i>S. greyana</i> <i>S. procumbens</i> Stout-stemmed; erect plant; 30+ cm tall with numerous hairy leaflets; large purple, blue, pink, or red pea-shaped flowers; long woolly seed pods; plants tend to grow from August to October especially after winter rains.	Swainsonism	Swainsonine (indolizidine alkaloid)	Subtropical and temperate regions of all states	Individual animals may develop a craving for the plants; horses especially selectively graze swainson pea; there are approximately 85 native <i>Swainsona</i> sp. in Australia; all should be regarded as poisonous until proven otherwise.	Swainsonine, which acts in two ways: blocks the enzyme mannosidase, which produces an acquired mannose lysosomal storage disease and interferes in the normal production of enzymes, hormones and immunoglobulins. Cattle: can graze the plant for several weeks before development of clinical signs; signs after ~6 weeks of grazing include paddling gait, hyperexcitability, loss of condition, charge when approached; clinical signs progress to depression, incoordination, some deaths; animals that survive are poor in condition; long-term effects include infertility and abortion. Toxidrome is reversible if exposure is up to 4 weeks. Horses develop clinical signs after ~2 weeks of grazing; initial signs include paddling gait, hypersensitivity to touch, tremor, forced respiration; these progress to depression, incoordination, head pressing, hind leg dragging, front leg high-stepping, recumbency, death; long-term effects include emaciation, difficulty eating, lowered head, ventral swelling, apparent blindness, walking in circles.	Toxidrome in horses resembles Birdsville disease ( <i>Indigofera linmaei</i> poisoning); animals may have concurrent access to both plants; Birdsville disease occurs only in horses, whereas swainsona poisoning occurs in both horses and cattle; swainsona poisoning is mostly a disease of late winter to early summer, whereas Birdsville disease occurs primarily in summer, particularly after the first summer rains; mannose lysosomal storage disease is histologically identifiable in swainsona poisoning, whereas there are no specific anatomic pathology changes associated with Birdsville disease.
Weir vine poisoning	<i>Ipomea calobra</i> Slender vine with heart-shaped leaves and trumpet-shaped pink flowers.	Swainsonism	Swainsonine (indolizidine alkaloid)	Maranoa district of QLD	As per swainsona poisoning.	As per swainsona poisoning.	As per swainsona poisoning.

Birdsville disease Birdsville indigo poisoning	<i>Indigofera linnaei</i> Low; spreading plant; thick taproot; numerous thin woody stems forming a mat up to 1.5m across; leaves fern-like; flowers are very small and occur in dense clusters in the forks of the leaves; flowers are red turning blue as the leaves dry out; seed pods are gray, very narrow, sharply pointed at the tip, and contain two cube-shaped seeds.	3-Nitropropionic acid toxicity in horses; indospicine poisoning in dogs fed raw or cooked meat from poisoned horses or from camels grazing <i>I. linnaei</i>	Indospicine (hepatotoxin); 3-nitropropionic acid (neurotoxin); horses are resistant to the hepatotoxic effects of indospicine; toxins are present in the leaves and seeds of the plant at all times (green or dry).	Widespread in subtropical and arid regions of WA; NT and QLD	Drought resistant and responds rapidly to rain; disease outbreaks commonly occur in spring or summer after rains because of the rapid response of the plant to moisture; up to 100% mortality in horses; under normal conditions, the plant does not cause clinical disease in cattle; raw and cooked meat from poisoned horses is toxic to dogs because of the indospicine – secondary poisoning may occur.	In horses, clinical signs can develop after 10 days of feeding; clinical signs include general weakness, nervousness, depression, incoordination, shivering, twitching, and swaying; neuromotor signs are more evident when the animal is, or has been, under physical stress; bad breath; toe dragging (tracks are characteristic) and excessive wear on the front of the hoof; continuous ingestion will result in death; neurological signs are irreversible; secondary indospicine poisoning in dogs is characterized by acute fulminant and sometimes fatal hepatic failure.	Can be confused with swainsona poisoning, and it is possible for the two diseases to occur concurrently; see swainsona poisoning.
Bitter bark poisoning Quinine tree poisoning Quinine bush poisoning	<i>Alstonia constricta</i> Tree to 12m height; suckers from the roots producing thickets; leaves are narrow, lanceolate, 5–20 cm in length; flowers white to cream, 2–4 cm diameter; bark has a corky textured; white sap.	Indole alkaloid poisoning in ruminants and dogs fed meat from poisoned animals	Indole alkaloids (alstonine; altonidine; reserpine); does not contain quinine (common name derived from the bitter taste of the bark); alstonine and reserpine are used as antipsychotic drugs.	Eastern Australia from the Tropic of Capricorn southward to northern NSW	Leaves and fruit are poisonous; poisoning usually occurs when there is a shortage of feed; meat from poisoned animals is toxic to dogs.	Tetanus-like toxidrome: excitability, nervousness, stiff legs, saw horse gait, staggers, redness of the ocular mucous membranes.	
Wax flower poisoning Waxvine poisoning	<i>Hoya australis</i> Evergreen climbing vine; shiny round succulent leaves; milky sap; clusters of white flowers 1.5–2.5 cm in diameter, with five thick, waxy, triangular petals; strong sweet scent and produce copious nectar.	Tetanus-like toxidrome	Unknown neurotoxin	Popular garden plant; natural distribution is from Grafton in northern NSW to Cape York; grows on the edges of rain forest and in rocky exposed habitat.	Unpalatable; poisoning usually occurs when there is a shortage of feed.	Tetanus-like toxidrome: incoordination, knuckling of fetlocks, tremoring, collapse of either fore- or hindquarters, recumbency, tetanic spasms; no effective treatment, although mildly effected animals may recover.	
Poison morning glory Australian morning glory poisoning	<i>Ipomoea muelleri</i> Slender vine with heart-shaped leaves and trumpet-shaped pink flowers.	Neurological toxidrome in ruminants and horses	Calystegines implicated	Northern Australia; found in open forest; margins of monsoon forest and vine thicket; garden plant	Often the only green feed at the start of the wet season and the end of the dry season.	Animals appear inebriated and develop hindquarters ataxia that may be permanent in cattle; associated with transient staggering toxidrome in sheep; affected animals appear during the wet season and die by the following dry season.	

(Continued)



TABLE 79.1 (Continued)

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Floodplain staggers Stewart Range syndrome Pacific bent grass poisoning Blown grass poisoning	<i>Agrostis avenacea</i> Native to Australia and New Zealand; invasive weed in California, Texas and Hawaii; tufted perennial grass to 65 cm tall; inflorescence is a panicle of wispy strands terminated by 2–3 mm long fuzzy spikelets; grass is most commonly found in flat low-lying areas that are flooded up to 30 cm during winter and in channels and depression where water collects.	Corynetoxin poisoning in ruminants, pigs, horses	Corynetoxins (tunicaminyluracils) produced by <i>Rathayibacter toxicus</i> in nematode ( <i>Anguina funesta</i> ) induced galls in the seed heads and crowns of the plant.	Southeastern SA, northern NSW	Toxin production is greatest when the grass is haying off; most outbreaks occur ~1 week after the start of haying off; bacteria is identifiable as a yellow slime on the seed heads that dries to an orange-colored material; nematode galls can be identified in the seed heads; unlike annual ryegrass toxicity, the plant can become infected two times per year; outbreaks occur with favorable weather conditions; hay from affected pastures is poisonous.	Resembles annual ryegrass toxicity: onset of toxidrome delayed up to 4–5 days; reluctant to move; uncoordinated gait; limb weakness; muscle fasciculations; stand with a backwards and forwards rocking motion; individual muscle movements rigid; irregular and abrupt; whole limb movements excessively pronounced; high stepping or “rocking horse” gaits; loss of hind limb control; sudden collapse while running; intermittent episodic convulsions; movement or exercise exacerbates the clinical signs; high mortality (up to 90%); triggers abortion; lambing and calving losses to 30%.	Resembles annual ryegrass toxicity; Identical to annual beard grass ( <i>Polypogon monspeliensis</i> ) toxicity.
Caustic bush poisoning Caustic vine poisoning Milk vine poisoning	<i>Sarcostemma australis</i> Leafless succulent scrambler or bush; with smooth, jointed, succulent stems; flowers are pale green to cream-white, star shaped and waxy; in clusters at the stem joints; sap is corrosive to skin and eyes.	Neurological toxidrome in sheep, cattle; toxic to horses	Unidentified neurotoxin; may resemble cynanchoside.	Northern Australia extending southwards to northern NSW	Moderately palatable; rarely grazed except in very dry seasons when fodder is scarce; fresh shoots may be more toxic than the old shoots.	Toxidrome consists of restlessness, staggering and collapse, rapid breathing, paddling legs, jaws may be clamped shut, excessive salivation, seizures, convulsions, limb paddling.	Plant can be confused with <i>Euphorbia sarcostemmoides</i> ; <i>Euphorbia</i> sp. in Australia produce acute GI irritation (and cyanogenic glycoside toxicity in some species).
Grass tree poisoning Called “wamps” in NT (after the sound that cattle make when they collapse and hit the ground)	<i>Xanthorrhoea johnsonii</i> <i>Xanthorrhoea fulva</i> Grass trees	Neurological toxidrome in cattle	Unidentified	Coastal and subcoastal eastern Australia	Flower spikes are the most toxic parts, but leaves have caused problems.	Toxidrome consists of transient spinal cord dysfunction with associated posterior ataxia and urinary incontinence; delayed onset after access may be up to 10 weeks; clinical signs: constant sideways lurching of hindquarters, fall easily, have difficulty rising, urinary incontinence, weight loss; toxidrome is reversible within 2–3 weeks after cessation of exposure in most cases.	

Byfield fern poisoning Zamia fern poisoning Zamia staggers	<i>Bowenia serrulata</i> <i>Bowenia spectabilis</i> Fern-like cycad; distinctive among cycads because they have bipinnate leaves.	Neurological toxidrome in cattle is most common disease observed.	Unidentified neurotoxin (neurotoxin possibly related to $\beta$ -methylamino-L-alanine and/or $\beta$ -oxalylamino-L-alanine); cycasin (a pro-toxin that is transformed by plants and intestinal flora to methylazoxymethanol, an inhibitor of protein synthesis).	QLD rain forest; located on protected slopes; usually near a stream.	Leaves are toxic and remain neurotoxic even when dried; <i>B. spectabilis</i> is a cultivated ornamental plant; cycasin is destroyed by drying of the plant, but the dried plant remains neurotoxic.	Clinical signs consist of hindquarters ataxia consisting of an unusual swaying motion of the hind limbs, flexion of the hock and fetlock, wobbling and malpositioning of the hind legs.	Resembles poisoning by other members of the cycadales order (cycads); i.e., zamia poisoning, lepidozamia poisoning, cycad poisoning; possibly similar to human amyotrophic lateral sclerosis/parkinsonism–dementia complex (Guam disease, lytico-bodig disease).
Zamia staggers Burrawang staggers	<i>Macrozamia</i> sp. Palm-like cycad with slender leaves; seeds red to yellow in male or female cones. <i>Lepidozamia</i> sp. Unbranched tall tree-like cycads with persistent leaf bases.	Neurological disease in cattle and liver disease in sheep are the most common presentations; horses and pigs are also susceptible.	As per <i>Bowenia</i> sp.	<i>Macrozamia</i> sp.: QLD and NSW; mainly in coastal areas; Bega to Macleay River; and westward toward Goulburn <i>Lepidozamia</i> sp.: rain forests of QLD and NSW	As per <i>Bowenia</i> sp.; may be found as a contaminant in chaff; cultivated as ornamental plants; all species are regarded as toxic unless proven otherwise.	A slowly developing, irreversible nervous paralysis of the hind legs similar to <i>Bowenia</i> sp. OR Clinical signs relate to chronic liver damage: loss of appetite, ill-thrift, diarrhea, chronic weight loss, eventual death due to hepatic insufficiency. Poisoning may eventually be irreversible.	As per <i>Bowenia</i> sp.: seed cones from <i>Macrozamia</i> sp. were used as a food source by indigenous Australians (notably the Cadigal) but require extensive processing to render them safe.
Corkwood poisoning	<i>Duboisia myoporoides</i> <i>D. leichhardtii</i> Small tree members of the nightshade family; cork-like bark; simple leaves; white tubular flowers in bunches at the branch ends; fruits are black and berry-like.	Deliriant anticholinergic tropane alkaloid poisoning	Tropane alkaloids including hyoscyamine and scopolamine	Coast and tablelands of NSW; central and coastal QLD; found in high rainfall areas near rain forests; up to 1000 m altitude; and also in rain forest regrowth areas	Toxic to cattle, sheep, horses, camels, pigs, dogs, and cats; generally unpalatable; rarely grazed except in very dry seasons when fodder is scarce; may be grazed by animals inexperienced with the plant; sheep somewhat resistant compared with other species; used in herbal remedies.	Classical deliriant anticholinergic toxidrome: that is, hyperthermia, tachycardia, bizarre and possibly violent behavior, severe mydriasis, photophobia, muscle trembling, muscular weakness, staggers, colic and scours, death within 12 h but may recover with no long-term effects; can be treated using physostigmine; mnemonic for the toxidrome: “blind as a bat; mad as a hatter; red as a beet; hot as hell; dry as a bone; the bowel and bladder lose their tone; and the heart runs alone.”	Resembles atropine and <i>Datura</i> sp. (e.g., jimson weed or Jamestown weed) poisoning; leaves are harvested commercially for production of scopolamine and hyoscyamine; workers handling the leaves have been affected with dry throat, headache, and loss of visual acuity; plant is a target for recreational drug users.

(Continued)

TABLE 79.1 (Continued)

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Pituri poisoning Pitchuri thorn apple poisoning Pitcheri poisoning	<i>D. hopwoodii</i> Erect shrub; 1–3 m high; long, narrow leaves; flowers are white, bell-shaped with violet-striped throats; flowers June to November; berries are black, 3–6 mm in diameter.	Nicotine poisoning	Nicotine; nornicotine	Arid interior regions of Australia	Toxic to cattle, sheep, horses, camels, pigs, dogs, and cats; generally unpalatable; rarely grazed except in very dry seasons when fodder is scarce; may be grazed by animals inexperienced with the plant; plants with high nornicotine:nicotine ratios are generally more poisonous.	Classical nicotine poisoning (depolarizing neuromuscular block): tremoring, weakness, stumbling and/or incoordination, depression or hyperactivity, tachypnea, respiratory insufficiency, excessive salivation, dilated pupils, apparent blindness, vomiting, diarrhea, colic, seizures, collapse, bradycardia or tachycardia; ruminants may bloat; some animals may be found dead with no history of neurological signs (presumably from lethal cardiac arrhythmias).	Dried leaves from plants in the Mulligan River area (high nicotine and low nornicotine content) were mixed with <i>Accacia</i> sp. ash and used by indigenous Australians as a form of chewing tobacco; in areas where the plants have a high nornicotine:nicotine ratio, the plants were used as an animal poison for food gathering.
Finger cherry poisoning	<i>Rhodomyrtus macrocarpa</i> Small tree; opposite broad leaves with oil glands; white flowers with five petals; red fruit.	Blindness	Unidentified	Northern QLD rain forest	Also cultivated as a garden plant.	Produces permanent blindness within ~24 h of consumption due to optic nerve degeneration.	Native guava causes a similar disease.
Native guava poisoning	<i>R. psidioides</i> Resembles <i>R. macrocarpa</i> except leaves are narrow, lance-shaped, and 25 cm long.	Blindness	Unidentified	Northern QLD rain forest		Produces permanent blindness within ~24 h of consumption due to optic nerve degeneration.	Finger cherry produces a similar disease.
Blind grass poisoning	<i>Stypandra</i> sp. Tufted perennial herb, with rhizome and fibrous roots; up to 1 m diameter; erect aerial stems with opposite leaves; small blue to white flowers, in spring.	Neuromotor disease plus blindness	Stypandrol	WA; eastern NSW, QLD, VIC, and TAS; forest and woodland	Plants vary in their toxic content; most toxic times seem to be when shoots are young and green and when plants are flowering.	High-stepping gait; rapidly developing hind leg weakness; difficulty in raising head; progresses to total paralysis; depression; permanent blindness, death; some animals may recover if exposure stopped.	<i>Dianella</i> sp. (blue flax lily) and <i>Hemerocallis</i> sp. (day lilies) cause similar problems.
Austral bracken fern poisoning; bright blindness in sheep; chronic hematuria syndrome in ruminants	<i>Pteridium esculentum</i>	Thiamine deficiency polioencephalomalacia; bright blindness in sheep; chronic hematuria syndrome in ruminants; immunodeficiency; chronic anemia/neutropenia; mutagenic and carcinogenic	Thiaminase; ptaquiloside; norsequiterpene glucocides	All states; particularly in wet areas with well-drained soils	Rhizomes are very toxic; poisoning develops after protracted feeding; often symptoms do not appear until days or weeks later; cattle and horses most affected, sheep less so; 2–4 weeks of intake required for toxicity.	Depressed bone marrow functions in cattle and sheep resulting in anemia, thrombocytopenia, neutropenia, and immunotoxicity; polioencephalomalacia; bladder cancer in sheep and cattle (chronic hematuria syndrome); progressive retinopathy in sheep (bright blindness).	Similar disease with mulga (rock) fern.

Mulga fern poisoning Rock fern poisoning; blindness in sheep	<i>Chelilanthes</i> sp.	As per bracken fern.	Thiaminase; norsequiterpine glucocides (particularly ptaquiloside)	Throughout Australia	Generally avoided but eaten in drought periods when there is little else available; low-level ingestion is safe, but too much is dangerous; fern-infested paddocks can be grazed safely if animals are moved after ~10 days and rested in fern-free areas for ~3 weeks.	Resembles bracken fern toxicity.	Bracken fern toxicity
Nardoo poisoning	<i>Marsilea drummondii</i> A water fern, or herb; short creeping rhizome; leaves have a slender stalk tipped with four wedge-shaped leaflets that float on the surface, or spread on dry land and look like a four-leaf clover.	Thiamine deficiency polioencephalomalacia; chronic nonresponsive anemia due to bone marrow depletion; immunotoxic; mutagenic/ carcinogenic; beriberi (human)	Thiaminase; norsequiterpine glucosides	Inland areas; in depressions or river flats subject to flooding; different <i>Marsilea</i> sp. found in all mainland states; all species should be considered potentially toxic; sporocarps are very drought-resistant, surviving up to 100 years in dry conditions.	Toxin levels highest in summer, lowest in winter; often grazed without ill effect, but if they are the only plants available, there will be overdosing and stock losses; mainly sheep and horses are affected; good fodder plant under normal conditions.	Typical polioencephalomalacia toxidrome in ruminants; chronic anemia/neutropenia/ thrombocytopenia; chronic hematuria/bladder neoplasia syndrome in cattle; was an important food source for indigenous Australians (Cooper Creek and the Yandruwandha peoples), who rendered the plant safe by cooking it into “cakes” called “padlu”; famous as a potential cause of death of the explorers Burke and Wills.	Other causes of polioencephalomalacia (e.g., dietary sulfur); other sources of thiaminase (e.g., bracken ferns).
Stinging tree Gympie stinger	<i>Dendrocnide</i> sp. Five species in Australia; large trees, but early stages and low, new growth are common sources of exposure; leaves are large, heart-shaped, with two rounded lobes at the base, margins may be toothed, and young leaves are covered with large, stiff, stinging hairs.	Extreme pain	Hemolytic saponins; morodoin; stinging hairs contain acetylcholine, histamine and 5-hydroxytryptamine.	Cape York to Kiama and Nepean area in NSW; grows on the edges of rain forests and in clearings	Generally not palatable, except for goats; simple contact with skin is extremely painful; horses are particularly vulnerable to the skin effects; leaves still dangerous when dried (remain dangerous for at least 40 years).	Contact with leaves of the plant is extremely painful; irritant toxins are injected through the skin by hollow plant hairs; pain is often intolerable, long-lasting, and recurrent for many weeks; horses often display a frenzied agony after skin contact with the plant; pain is difficult to control or treat, particularly in horses.	

(Continued)



TABLE 79.1 (Continued)

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Georgina gidgee poisoning	<i>Acacia georginae</i>	Fluoroacetate poisoning	Organic fluoroacetates	Northwest QLD and adjacent eastern NT	Toxic to most animals (with the exception of some native Australian marsupials); most deaths occur during the dry season; most dominant animals are most likely to be affected; meat from poisoned livestock is poisonous to dogs and cats.	Death within minutes of first clinical signs; animals often found dead; tachypnea; trembling; sudden heart failure; cardiac arrhythmias; bloat; frequent urination.	1080 poisoning; 1080 is the sodium salt of monofluoroacetate; the number "1080" was the original catalog number for the substance; identical toxidrome to poison pea ( <i>Gastrolobium</i> sp.) toxicity; several other Australian native plant genera contain the toxin: <i>Gompholobium</i> sp., <i>Oxylobium</i> sp., <i>Nemcia</i> sp.
Poison pea toxicity; desert poison bush; heart-leaf poisoning; wallflower poisoning; and numerous other species and/or location-specific names	<i>Gastrolobium</i> sp. More than 100 Australian native species; leafy shrub to 2 m high; dull-green opposite leaves, notched at the tip; leaves are thick and stiff; several stems arising from lignotubers; flowers are pea-shaped, dark red to purple.	Fluoroacetate poisoning	Organic fluoroacetates; one of the most toxic plants in Australia.	Southwest region of WA	As per Georgina gidgee poisoning; mostly affects sheep; plants are most toxic when flowering; flowers and seeds are highly toxic; suckers are very poisonous; leaves are less palatable and less toxic; most species are potentially toxic, although toxicity is often intermittent and seasonal; goats have been used to control the plant, but with some animal losses.	As per Georgina gidgee poisoning.	As per Georgina gidgee poisoning.
Billy button poisoning Plains plover daisy poisoning	<i>Leiocarpa (Ixiolaena) brevicompta</i> Low growing, branched, annual or short-lived perennial; woody base; woody stems with white cottony hairs; green narrow leaves with no stalk; circular single yellow flower head with basal bristles; multiple angular hairless seeds.	Striated muscle myopathy	Crepenynic acid and other toxic unsaturated fatty acids	Common in inland Australia, from central QLD to central NSW to SA	Mostly affects sheep; lambs are usually the first affected; only mature dried seed heads are poisonous; toxins are concentrated in the seeds.	Toxidrome occurs within 2 weeks after start of grazing; sudden collapse and death during herding, exercise intolerance, muscle weakness; short strides; staggering; hind limb collapse; cardiac arrhythmias; hyperthermia, tachypnea; movement and mustering exacerbate the toxidrome; treatment with selenium may be helpful in some cases.	Can be confused with white muscle disease (nutritional muscular dystrophy, vitamin E/selenium deficiency).

Cyanogenic glycoside poisoning	<i>Brachyachne</i> sp. (native couches) <i>Eremophila maculate</i> (spotted emu bush) <i>Eucalyptus cladocalyx</i> (sugar gum) <i>Euphorbia</i> sp. (some species) <i>Heterodendron oleifolium</i> (rosewood) <i>Lotus</i> sp. (birdsfoot trefoils)	Acute cyanide poisoning	Cyanogenic glycosides	Most areas of Australia contain a cyanogenic glycoside-containing plant species.	Ruminants generally more susceptible; consumption of water following ingestion of plants increases the hydrolysis of cyanogenic glycosides to cyanide; the following generally increase the cyanide content of the plants: damage to plant, young rapid growth, seeds, regrowth after cutting, cool moist growing conditions, nitrate fertilization.	Sudden death with few clinical signs is the most common toxidrome; animals may have “cherry red” venous blood due to high venous pO <sub>2</sub> ; “cherry red” venous blood is often a necropsy finding, hence “cherry-red equals dead.”	
Nitrate poisoning	<i>Dactyloctenium radulans</i> (button grass)	Acute nitrate poisoning	Nitrate	Widespread native pasture grass in all states	Dangerous only when heavily fertilized.	Classical nitrate poisoning	
Pimelea poisoning	<i>Pimelea trichostachya</i> <i>P. simplex</i> <i>P. elongata</i> Annual herbs; opposite leaves on multiple branches; branches end in a flower spike with many flask-shaped yellow-green flowers.	Chronic right-sided heart failure, anemia and persistent diarrhea in cattle only	Simplexin (diterpenoid)	Inland northeastern regions; often in disturbed areas	Unpalatable; poisoning usually after the plants die and contaminate other pasture plants or hay; poisoning more likely after winter rains.	In cattle only, produces chronic right-sided heart failure, anemia, and chronic diarrhea; produces only diarrhea in other species.	
Cardiac glycoside poisoning	<i>Adonis microcarpa</i> <i>Asclepias curassavica</i> <i>Thevetia peruviana</i> <i>Cryptostegia gandiflora</i> <i>Glyceria maxima</i> <i>Gomphocarpus</i> sp. <i>Homeria flaccid</i> <i>H. miniata</i> <i>Bryophyllum</i> sp.	Cardiac glycoside poisoning	Cardiac glycosides	Most regions of Australia have at least one cardiac glycoside-producing plant; many are introduced plants that have been important causes of stock losses.	Generally not palatable; often only grazed during dry seasons when fodder is scarce; may be grazed by animals inexperienced with the plants; potential contaminants of hay.	Typical cardiac glycoside poisoning; may show signs of GI distress (e.g., scouring) due to other irritants present in the plant.	Camel poisoning
Camel poison	<i>Erythrophleum chlorostachys</i> (Cooktown ironwood) Large tree; smooth white or yellow-brown bark; discolorous leaves; white flowers; barrel-like fruit.	Cardiac glycoside-like poisoning	Diterpenoid alkaloids and cinnamic acid derivatives	Northern Australia; open woodland	All parts of the plant are toxic; aggressively suckers and suckers are toxic and accessible to grazing animals; affects ruminants and horses.	Resembles cardiac glycoside poisoning.	
Cattle bush poisoning Whitewood poisoning	<i>Atalaya hemiglauca</i> Small to medium sized tree; gray bark; leaves alternate, varying shape, dull gray, or bluish green; flowers are cream green, in large panicles; very hardy and drought resistant.	Cardiac failure syndrome particularly in horses and to lesser degree in ruminants	Unknown	Inland WA, QLD, NT, western NSW	Young shoots and fruit are toxic; poisoning occurs when plant forms a large part of the diet; high palatability; used as a drought fodder for cattle but should not be fed to horses.	Either no effects or death; in horses: vague signs of distress, staggers, severe swelling of the head, muscle weakness, myoglobinuria, followed by fairly rapid death; staggers in cattle.	

(Continued)

TABLE 79.1 (Continued)

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Poison sedge	<i>Schoenus asperocarpus</i> Tufted perennial; grass-like or herb (sedge); 0.15–1 m high.	Pneumotoxin	An isoprenyl guanidine: galegine	Southwestern WA		Toxin affects pulmonary vascular permeability; acute massive pulmonary edema and thoracic effusion.	
Stinkwood poisoning; panting disease	<i>Zieria arborescens</i> Tall shrub or small tree; to 4 m high, 3 m across; branches covered with star-shaped hairs; highly aromatic; fast growing; leaves have unpleasant odor when crushed.	Pneumotoxin	Unknown	TAS, VIC, southeast NSW, wet forest areas		Acute massive pulmonary edema and emphysema	
Soluble oxalate poisoning	<i>Neobassia proceriflora</i> (soda bush) <i>Portulaca oleracea</i> (inland pig weed, munyeroo) <i>Salsola kali</i> (soft roly-poly) <i>Setaria sphacelata</i> <i>Chenchrus ciliaris</i> (buffel grass) <i>Oxalis</i> sp. <i>Rumex</i> sp. <i>Acetosella vulgaris</i> <i>Trianthema</i> sp.	Calcium oxalate tubular nephrosis; hypocalcemia; <i>C. ciliaris</i> is noted for producing secondary nutritional hyperparathyroidism in horses ("big head").	Soluble and absorbable oxalates	Most areas of Australia have at least one soluble oxalate-containing plant.	Typically, the plants are more palatable when young.	Typical soluble oxalate acute hypocalcemia + crystalline tubular nephrosis toxidrome; chronic renal nephrosis toxidrome	Milk fever (post-parturient hypocalcemia)
Alkali disease; blind staggers	<i>Morinda reticulata</i> (mapoon, adaa) <i>Neptunia amplexicaulis</i> (selenium weed)	Selenium toxicity: alkali disease and blind staggers	Selenoamino acids in <i>M. reticulata</i> ; ethanol-soluble selenium compounds in <i>N. amplexicaulis</i>	<i>M. reticulata</i> , Cape York; <i>N. amplexicaulis</i> , central QLD	Cases often occur after burning back of scrub and in the spring.	Classical alkali disease and blind staggers; alkali disease is more common.	
Poison sage; lamb poison	<i>Isotropis</i> sp. Shrubs or herbs; pea-type flowers; flowers are purple, yellow, or orange.	Irreversible nephrosis	Iforrestine	Not found in VIC or QLD	Most toxic when flowering.	Anorexia; anuria; renal failure; gastroenteritis; sudden death can occur.	
Gastroenteritis (partial list)	<i>Castenospermum australe</i> (black bean tree) <i>Euphorbia</i> sp. <i>Stemodia kingii</i>	Acute and severe gastroenteritis	Cucurbitacins in <i>Stemodia kingii</i>	Most areas of Australia contain plants capable of triggering acute gastroenteritis.		Acute, often severe, diarrhea	

NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia.

TABLE 79.2 Summary of major New Zealand plants that are hazardous to livestock

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Strathmore or Pimelea or New Zealand Daphne poisoning	<i>Pimelea prostrata</i> Small, prostrate, branched woody shrub; to 50 cm in diameter; branches are scarred, dark brown to black; leaves pointed oval, greenish-gray, ~5 mm long; waxy white flowers, 3–4 mm long, red or white ovoid berries.	Irritant (mucous membranes and skin); hypersensitivity; strong effect on cardiovascular function	Prostratin (dipterene acetate) in the leaves and bark	North and south islands; grasslands; open shrubland; along river terraces; up to an altitude of 1500 m	Poisonings more common in winter when feed is restricted. Formerly a toxicity of primarily horses, it is now reported more often in cattle; sheep are resistant to poisoning.	Horses: profuse watery diarrhea, blistering and irritation of oral mucous membranes and eyes, depression, loss of appetite, muscular pain, strong disinclination to move; necropsy features include gastric ulceration and gastroenteritis, fatty degeneration of liver; toxidrome in cattle resembles that of horses.	Differentials include <i>Pieris japonica</i> poisoning, causes of jaundice (e.g., facial eczema), allergic hypersensitivity reactions, bacterial or viral enteritis, systemic bacteremias, and viremias including foot and mouth disease.
Tutu toxicity	<i>Coriaria arborea</i> Leaves opposed, vary from narrow or filiform to ovate to lanceolate; flowers axillary or terminal racemes; fruit usually black or purple.	Tetanus-like with CNS excitation (blocking of spinal glycine and CNS GABA <sub>A</sub> receptors)	Tutin (lactone from the picrotoxin group); localizes in the CNS, causing medullary excitatory poisoning.	Throughout New Zealand; often found in stony areas	Toxicity usually in hungry or starved cattle and occasionally sheep; all parts are poisonous except the petals surrounding the seeds and the roots; toxin is concentrated in leaves (particularly young seeds); horses are not attracted to tutu; circus elephants have been poisoned.	Picrotoxin-like: salivation, nausea, excitement, convulsions, coma, and death; sudden onset 24–48 h after ingestion; cattle may exhibit blind charging, muscle twitching, extreme excitement, epileptiform convulsions, bloating, and regurgitation; produces amnesia in humans; stock losses of 5–10% of beef herds in the South Island high country; honey produced from tutu is toxic.	
Ngaio toxicity	<i>Myoporum laetum</i>	Hepatogenous damage and photosensitization	Ngaione and several other furanosesquiterpenoid essential oils. Ngaione is a protoxin that requires hepatic metabolic activation.	Common in coastal areas and lowland forests as far south as Otago; common in gardens	Toxicity of individual plants varies; leaves normally most toxic part; serious losses in cattle, sheep, pigs, and horses; most poisonings occur after storms blow down tree branches or when clippings are thrown out where stock have access to them.	Hepatogenous secondary photosensitization; recovery among survivors is very slow; severe constipation, abdominal pain, dullness, loss of appetite, no rumination; occasionally icterus.	Exposure to sporidesmin (facial eczema), ragwort, microcystis or nodularin (algal blooms), and other hepatotoxic compounds may exacerbate Ngaio toxicity.
Karaka poisoning	<i>Corynocarpus laevigatus</i> Tree to 15 m tall; leaves oblong, 10–15 cm long, dark green, thick, and glossy; flowers are small, yellow green; orange fruit, 25–40 mm long with three layers (outer skin, fleshy layer, and hard inner layer enclosing the seed).	Neurological problems; paralysis	Karakin (glucose ester of 3-nitropropionic acid)	Lowlands, especially the coasts; abundant in the North Island; found in Nelson, Marlborough, and Banks Peninsula in the South Island	Risk of ingestion is higher in autumn; fruit and seeds are main toxic parts; fruits remain toxic for months; fruit used as food by the Maori but require extensive processing to render them safe; nectar is toxic to bees.	In cattle, paralysis of the hind limbs and an end to lactations may occur. In dogs, convulsions and spasms may precede paralysis of the hind limbs and muscle incoordination. Death may occur. A goat that ate karaka berries showed pain, depression, and diarrhea.	

(Continued)



TABLE 79.2 (Continued)

Common name of disease	Species and identification	Diseases	Toxins	Distribution	Risk factors	Toxidrome	Association with other diseases
Kowhai toxicity	<i>Sophora sp.</i> Twenty-seven (very similar) species; trees or shrubs to 20 m tall; leaves are $\geq 15$ cm in length, with each bearing 20–40 pairs of leaflets; flowers ostentatious yellow in drooping racemes or panicles in spring; pods are long and winged; seeds yellow.	Stimulation of respiration; excitation of muscle; paralysis of peripheral sympathetic ganglia	Cytisine (acts like nicotine); also anagryne, matrine, <i>N</i> -methylcytisinesophoramine, and sophochrysine	Throughout New Zealand in open forests, along forest margins, riverbanks, and in open damp or rocky places; commonly grown as an ornamental	All parts are poisonous; most poisonings are caused by seeds; children, livestock, and bees have been poisoned by seeds, plant material, or nectar, respectively; all species are toxic until proven otherwise.	Resembles nicotine poisoning; recumbency, ataxia, tachycardia, increased respirations, abdominal pain, and hypomotility have been reported; death is due to nicotinic stimulation of the nervous system.	
Tree nettle toxicity Ongaonga poisoning	<i>Urtica ferox</i> Bush to 2 m tall; plants are much branched; stinging hairs up to 6 mm long covering the stalks and margins of the leaves; flowers are small and green.	Nervous and gastrointestinal signs as well as local irritation	Histamine, serotonin; acetylcholine, and other unidentified pain-producing agents; toxins are concentrated in the stinging hairs.	Throughout New Zealand and abundant; particularly on waste and neglected land; extend south to Otago	Contact with plant causes toxicity; all species susceptible.	Horses: excitable and restless; lesions can be seen in sheep on the udder and hairless parts. Dogs: trembling, vomiting, difficulty breathing, and general pain; convulsions and death may follow in severe cases; temporary loss of scenting ability in dogs; survivors have muscle pain for several days.	
Poroporo toxicity Bullibul; Bullibulli; large kangaroo apple (Australia)	<i>Solanum laciniatum</i> and <i>S. aviculare</i> Soft wooded shrub to 3 m tall; leaves narrow, lanceolate, 15–30 cm long; flowers blue-purple; fruit pale yellow or orange berry.	Irritant, hemolytic, CNS depression with cardiac and respiratory failure	Solasonine, solamargine, and $\beta$ -solamargine	Lowland forest margins and shrublands throughout New Zealand	Not normally palatable; all parts are poisonous; toxins concentrated in unripe berries; fully ripe fruit are probably not toxic; poisoning associated with ingestion of green fruit and plant material by hungry or naive stock.	Profuse diarrhea with abdominal pain, depression, weakness and incoordination, trembling, rapid respiration and heart rate, dyspnea, excessive salivation, nasal discharge, and jaundice; sudden death.	
Bushman's friend Rangiora toxicity	<i>Brachyglottis repanda</i> Shrub or small tree; to 6 m height; branches are covered in soft white hairs; leaves ovate, 10–25 cm long, obvious veins; dull gray/green above and covered with white hairs on the underside; flowers white to creamy white in large drooping panicles.	Hepatogenous pyrrolizidine alkaloid (PA) toxicosis	Senkirkine and other PAs	Abundant in the North Island and found as far south as Kaikoura and Greymouth in the South Island	PAs most concentrated in young growing tips, leaf petioles, and in the cortex of thin stems; PAs not present in the flowers and only in low concentrations in the mature leaves; all species susceptible, although sheep somewhat resistant.	Senkirkine causes irreversible hepatic damage and altered vitamin A metabolism; typical PA toxidrome.	Other liver toxicants, such as sporidesmin, copper, phosphorous, iron, and phenolics, increase the risk of toxicity by PAs.
Buttercup poisoning Waoriki poisoning	<i>Ranunculus rivularis</i> (syn. <i>amphitrichus</i> ) Perennial, creeping, hairless buttercup; it prefers wet places.	Gastrointestinal tract irritant	Ranunculin (protoanemonin)	Ubiquitous; most poisonings in the North Island	Most toxic during flowering; hay containing the plant is safe when dried.	Severe gastroenteritis; milk has taste taint	

industry and the arid zone and semi-arid zone sheep industries. The following are approaches to reduce the risks associated with these circumstances:

- Mechanical methods of plant removal: Because of the sheer physical size of livestock properties in the semi-arid and arid regions of Australia, such methods are often impractical. However, on a small scale, such techniques may be valuable. A notable use of physical removal of poisonous plants over relatively large land areas is the use of “scalping” in the wheat belt of Western Australia. Scalping involves the removal of all topsoil that may contain *Gastrolobium* sp. seeds and replacing the topsoil with new poison pea-free soils sourced from elsewhere. This extreme and expensive technique has been historically used with some success.
- Use of herbicides: Again, because of the sheer physical size of livestock properties in the semi-arid and arid regions of Australia, such methods are often uneconomic. Herbicide use may, in fact, increase the toxicity (and in some cases the palatability) of some plants (e.g., the notable association between the use of some herbicides and nitrite poisoning).
- Use of biocontrol agents: A number of biocontrol agents are being developed in Australia for the control of introduced poisonous plants, notably for Patterson’s curse. Biocontrol agents are unlikely to be developed for indigenous plants.
- Sheep, cattle, and goats are somewhat more tolerant of some poisonous plants than are other livestock. The use of goats to control *Gastrolobium* sp. and the use of sheep on pastures containing heavy growths of Patterson’s curse and heliotrope are common examples. However, some stock losses are often encountered in these types of enterprises, and they require careful management.
- Supplementary feeding (including the use of safe but not normally consumed native plants) during drought is also regularly and successfully used to avoid the effects of drought and poisonous plants on livestock. This has led to the common practice of “droughtlotting” – the creation of temporary feedlots in order to maintain key livestock. Registers of safe native plants that can be used for drought feeding are commonly maintained by the various relevant state government departments. The leaves of *Acacia aneura* (mulga) have historically been extensively used as drought feed for sheep in the arid, central zone of Australia. Although the plant is effective for this purpose, consumption results in nutritional lipofuscinosis of the liver and kidneys of sheep (commonly called “black liver”). The pigment accumulation is benign and the animals suffer no adverse effects, but the liver and kidneys from affected animals cannot be sold for human consumption for aesthetic reasons, resulting in some financial loss.
- A long-standing and well-proven strategy to reduce the risk of stock losses due to drought conditions and/or when poisonous plants are present has been to physically relocate livestock from properties/regions that are entering into drought conditions to geographic areas where there are better prevailing conditions. This technique was pioneered by S. Kidman and Company cattle company in the early 1900s. Kidman, whose cattle company at one time owned 3% of the Australian landmass, and others developed and expanded a network of connected stations and traveling stock routes (TSRs) stretching across northern Australia, Queensland, and New South Wales to South Australia near the Flinders Ranges and also across New South Wales. Kidman could grow and fatten the cattle on the remote stations in the north and bring them down the lines of stations and TSRs to markets in the south, providing good feed and water during transport and avoiding the effects of drought on his livestock. TSRs (known colloquially as “long paddocks”) in Australia are authorized thoroughfares for the walking of domestic livestock such as sheep or cattle from one location to another. TSRs are a critical source of feed for livestock during drought conditions. Traveling stock on a TSR must travel 6 miles a day (by law) in order to avoid the grazing off of all fodder within a particular TSR area. However, the use of TSRs under drought conditions is not foolproof. There have been many notable large-scale stock losses on TSRs due to animals that are unfamiliar with a particular geographic area grazing the local poisonous plants.
- Another long-standing and well-proven strategy to reduce the risk of stock losses due to the rapid emergence of indigenous poisonous plants following a break in a drought is to not allow livestock into a grazing area until safe fodder plants dominate the pasture. Again, the ability to geographically relocate stock into safe areas and hold them there until the pastures return to a good and safe condition, as well as knowledge of the local flora and physical inspection of the pastures, is critical to the success of such a strategy.
- Good pasture management is often a key factor in reducing the impact of many poisonous plants. Critical factors include not overgrazing pastures, not overstocking pastures, and pasture spelling/rotation.

Another common problem in Australia has been the introduction of toxic weeds into a hitherto clean area. Effective integrated weed management programs are critical to reducing risks of this nature.

One of the important animal-related factors that are amenable to human intervention is to ensure that

livestock are familiar with the local fauna. Large-scale stock losses that have occurred when naive stock are moved or released into a new geographic area are commonplace in the history of poisonous plants in Australia. Limited, staged, and well-controlled introduction of naive livestock into a new geographic area may reduce the risk of such events. It is also critical to ensure that the new geographic area has plenty of safe forage and that relevant poisonous plants do not dominate the edible local flora. Particular care should be taken to ensure livestock are not hungry when they are introduced into a new area.

Another animal-related factor that is amenable to human intervention is boredom in horses. Horses that are not active or have low levels of environmental stimulation may indiscriminately graze on plants simply due to boredom.

## CONCLUSIONS

Based on Australian Bureau of Agricultural and Resource Economics (ABARE, 2006) economic data, the livestock industries of Australia generated approximately \$AU 15.4 billion for the 2004–2005 financial year, representing approximately 56% of the income from the top 10 Australian agricultural commodities by value. Unfortunately, recent economic data on the impact of poisonous plants on the Australian livestock industries appears to be lacking, but the current anecdotal estimate is approximately \$AU 100 million per year, or approximately 0.6% of the total income generated from the Australian livestock industries in 2004–2005. Poisonous plants continue to be an important cause of financial loss within the livestock industries in Australia.

Compounding the pure financial losses is the fact that “outbreaks” of plant poisonings are often tied to climatic conditions, such as droughts and floods, when producers are focused primarily on maintaining the minimal core herds of livestock that will allow them to rapidly restock and take advantage of good climatic and pasture conditions when they return. Currently available practical techniques are partially successful in reducing livestock losses, although well-proven techniques such as geographic relocation of livestock are economically expensive and capital intensive.

Historically, the investigation of plant poisoning in the Australian and New Zealand livestock industries has generally focused on death or severe acute events as key endpoints. There is little doubt that within these contexts, there is still much to learn regarding the poisonous plant flora of Australia and New Zealand. However, sub-clinical losses (e.g., diminished growth rates rather than outright stock losses) and the use of indigenous plants as safe sources of fodder (particularly during adverse climatic conditions) are also likely to be areas of future investigation.

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# Cyanogenic plants

Steven S. Nicholson

## INTRODUCTION

Cyanide, hydrocyanic acid, HCN, and prussic acid are terms relating to the same toxic principle. Cyanide is used as a fumigant and in chemical synthesis. Fifty to 60 parts per million (ppm) in air, as in fumigants, may cause poisoning. Cyanide salts are used in metal cleaning, hardening, refining, and in the recovery of gold from ores. Jewelers use potassium cyanide to clean products (Coentrão and Moura, 2011). Tailings and wastewater from recovery of gold from ores is a potential threat to wildlife. Wildlife deaths associated with cyanide-bearing mine waste solutions have plagued the gold mining industry for many years, but there is little published data showing the relationship between wildlife mortality and cyanide toxicity. A gap in knowledge exists with regard to monitoring, understanding the causal relationships, and managing risks to wildlife from cyanide-bearing waste solutions and tailings (Donato *et al.*, 2007). Burning nitrogen-based polymers used in plastics, fabrics, and seat covers releases HCN, which along with oxygen depletion, carbon monoxide, and other compounds, contributes to fire smoke toxicity (Alarie, 2002). Cyanide blocks molecular oxygen transfer in cytochrome oxidase systems in mitochondria causing tissue anoxia. The process is reversible. Various cyanogenic glycosides, which can hydrolyze to form hydrogen cyanide, are present in a number of plant species. These compounds probably developed as a defense against excessive grazing by herbivores. Only a few of these plants are a significant risk to livestock. Some are grasses cultivated as forage for livestock and horses, and others are ornamentals, commercial fruit trees, shrubs, weeds,

and range plants. All animal species are susceptible to cyanide poisoning. The ability of rumen microbial flora to rapidly hydrolyze cyanogenic glycosides makes ruminants particularly at risk of cyanide intoxication from plant sources. A review of the risks and effects of cyanogenic plants in animals was published by Burrows and Tyrl (2001).

## BACKGROUND

In general, the location of cyanogenic glycoside in plants is the epidermis, with highest levels in seeds, leaves, bark, and twigs, and lowest levels in fruit. Seeds of grain sorghum and other grasses do not contain these glycosides. Seeds of members of the Rosaceae family, including apple (*Malus* spp.), cherry (*Prunus* spp.), peach (*Prunus persica*), and apricot (*P. armeniaca*), do contain cyanogenic glycosides. Laboratory test results of plant tissues for the presence of cyanide are reported as cyanide potential because free cyanide is not present in plants but is generated from glycoside during testing.

HCN potential of cyanogenic plants ranges from a few parts per million to 8000 ppm dry weight from the glycoside dhurrin in foliage of a grain sorghum (Burrows and Tyrl, 2001). Similar levels may occur in Johnson grass (*Sorghum halepense*), which is considered a weed but also utilized for grazing and hay. Related sorghum hybrids and sudangrass forages have been developed that have less cyanide potential, but most may be a hazard under certain conditions. Bermudagrass (*Cynodon* spp.) and other grasses and clovers are known to produce



cyanogenic glycosides in immature forage. African star grass (*Cynodon nlemfluensis*) is an example of one of the species grown in the tropics and Florida that can cause a cyanide problem. Nitrogen fertilization increases glycoside content. Cyanide potential in these forages is greatest during early growth. Concentrations of the glycoside prunasin in leaves of *Prunus virginiana* may be as high as 6% dry weight (Majak *et al.*, 1981). During a growing season, glycoside levels in plants are generally highest in early growth and decline as maturity approaches. Fluctuations in glycoside levels during a growing season occur associated with climatic changes such as periods of drought. Shortages of forage caused by a 2-year drought in Kansas were further exacerbated by increases in nitrate and cyanide in the feed based on analyses of samples submitted to a laboratory (Pickrell *et al.*, 1991).

Physical damage to plant tissue (freezing, crushing, macerating, cutting, and drying) allows plant enzyme  $\beta$ -glycosidase to come in contact with and hydrolyze the glycoside to hydroxynitrile and free volatile HCN. The first step in this process yields a sugar and an aliphatic or aromatic  $\alpha$ -hydroxynitrile aglycone (cyanohydrin); then, formation of an aldehyde or a ketone, such as benzaldehyde, and hydrogen cyanide occurs (Conn, 1979). Generally, hay loses most of the HCN prior to feeding. In some situations, toxic levels may remain in large bales of *Sorghum* spp. when the cut forage dries rapidly and is immediately baled. Properly ensiled silage loses cyanide potential. Green chopping immature sorghum forage and feeding it the same day to ruminants is a serious potential hazard.

## PHARMACOKINETICS/ TOXICOKINETICS

HCN is rapidly absorbed from the gastrointestinal tract, lungs, and slowly through the skin. Acid stomach contents in monogastric animals limit the hydrolysis of cyanogenic glycosides and release of HCN. The rumen microflora rapidly hydrolyzes cyanogenic glycosides, releasing HCN, which is quickly absorbed and distributed to the tissues. Lower ruminal pH in cattle fed high-grain diets reduces the action of microbial enzymatic activity and release of HCN (Majak *et al.*, 1990).

## MECHANISM OF ACTION

Cyanide combines with the ferric ion in mitochondrial cytochrome oxidase, preventing electron transport in the

cytochrome system and thus blocking oxidative phosphorylation and ATP production. The effect is cellular hypoxia or histotoxic anoxia. This is a reversible action in sublethal doses or when successfully treated. The inhibition of oxidative metabolism puts increased demands on anaerobic glycolysis, which results in lactic acid production and may produce severe acid-base imbalance. The central nervous system is particularly sensitive to hypoxia, and exposure to toxic levels of hydrogen cyanide generally produces symptoms within minutes.

Arterial blood is normally bright red because of the presence of oxyhemoglobin. In cyanide toxicosis, oxygen is not released from oxyhemoglobin to the tissues and the bright red color remains in venous blood. This process, from ingestion of a toxic dose of plant material to release of cyanide in the rumen and onset of clinical signs, can occur within a few minutes.

Some cyanide is detoxified by an endogenous thiosulfate limiting process. Thiosulfate combines with cyanide to form thiocyanate, which is excreted in the urine. The reaction is catalyzed by the enzyme rhodanese. The ability to detoxify cyanide allows animals to safely metabolize small amounts of cyanide.

## TOXICITY

Ruminants are more likely to be poisoned by plant-origin cyanide than are other animals because rumen microorganisms readily release cyanide from the glycoside. An active microbial flora in the gut allows considerable but somewhat delayed and slower hydrolysis of cyanogenic glycosides in humans and hamsters and lesser amounts in mice, rats, guinea pigs, and monkeys (Stavric and Klassen, 1980; Adewusi and Oke, 1985; Frakes *et al.*, 1986). Although uncommon, there are reports of cyanide toxicosis in horses, pigs, and dogs. Cyanide poisoning possibly killed two adult wether alpacas that ate a garden-cultivated variety of South African daisy (*Osteospermum ecklonis*) with a cyanide potential of 6800 mg HCN/kg dry matter (McKenzie *et al.*, 2004). Eating raw plant material containing  $\beta$ -glycosidase along with crushed apricot, cherry, peach, or apple seeds has proved fatal in humans, dogs, and cattle. In general, monogastric animals including horses are poisoned by 1–3 mg/kg body weight (BW) of preformed HCN or cyanide salts. The lethal dose of sodium cyanide, 3 or 4 mg/kg BW to ruminants (Burrows and Way, 1977), is quite similar to the lethal dose of cyanide from the glycoside prunasin in plant material, 5 mg/kg BW in cattle (Majak *et al.*, 1990). Plant material containing more than 20 mg per 100 g (200 ppm) cyanide potential is considered hazardous. A level of 500 ppm has been used more

specifically for the sorghums (Burrows and Tyrl, 2001). The effects are not cumulative. One-half the lethal dose can be given repeatedly during the course of a day such that a total dose of four or five times the single lethal dose does not induce clinical signs. Tolerance does not develop (Burrows and Tyrl, 2001).

Eight Alpine cross-bred female goats were divided into two equal groups and were treated with ground frozen cassava leaves at a target dose of 6.0 mg HCN/kg/day by gavage for 30 consecutive days. Clinical signs were observed in all goats treated with cassava on the first day of the experiment. From the second day, the dose of cassava leaves was reduced to 4.5 mg HCN/kg/day. No changes were found in the blood chemical panel. A mild increase in the number of resorption vacuoles in the thyroid follicular colloid, slight vacuolation of periportal hepatocytes, and spongiosis of the mesencephalon were found in goats treated with cassava (Soto-Blanco and Górniak, 2010).

Death may occur within minutes after ingestion of a toxic amount of a plant containing high cyanide potential. An exception may occur when ruminants grazing arid rangelands ingest a toxic amount of chokecherry or arrowgrass while rumen contents are quite dry and release of HCN by rumen flora is delayed until the animal drinks water later in the day. Affected animals may be found dead in or near the water source.

Cattle may become apprehensive and excitable at the sight of herd mates that are suddenly affected and collapsing. Onset of clinical signs is peracute and includes apprehension, pronounced polypnea, and then dyspnea because initially there is stimulation of chemoreceptors in the carotid body and respiratory centers. The pupils dilate, and mucous membranes may be pink and venous blood a bright red, like that of normal arterial blood. Weakness, voiding of urine, collapse, paddling, and death follow within a few minutes. Sublethal cases may recover within 1 h.

Lesions of cyanide toxicosis are few. Mucous membranes may be pink, and venous blood may be bright red and clot slowly. Subendocardial and subepicardial petechial and ecchymotic hemorrhages typical of an agonal death may be present. A bitter almond or "cherry coke" odor from stomach contents is detectable in some cases. Venous blood may not be bright red in animals that have been dead for several hours. At necropsy, blood of animals that died of some other cause may develop bright red color when exposed to the air for several minutes.

Differential diagnosis of cyanide toxicosis in ruminants may include acute toxicoses caused by insecticides, nitrate and nitrites, urea and ammonia, ipomeanol, perilla ketone, 3-methylindole, blue-green algae toxins, and electrical shock or lightning strike.

When cyanide toxicosis is suspected, one should submit to the laboratory, along with the usual specimens,

refrigerated heart or skeletal muscle and rumen contents for cyanide ion detection. In blood, concentrations of 1 ppm or higher are consistent with severe intoxication in mammals and birds (Burrows and Tyrl, 2001). Blood should be kept in airtight containers at 4°C (Egekeze and Oehme, 1979). Continued hydrolysis of glycoside and loss of HCN may make necropsy samples less useful for confirming the diagnosis. Specimens can be immersed in mercuric chloride to prevent hydrolysis from continuing. Rumen or stomach contents can be examined for the presence of material from cyanogenic plants. For example, black cherry (*Prunus serotina*) intoxication in a goat was diagnosed by identification of leaf fragments in rumen contents (Radi *et al.*, 2004). A sensitive field test using alkaline picrate-treated filter paper strips can be prepared for testing plant materials and fresh rumen contents (Burrows and Tyrl, 2001).

Chronic cyanide poisoning (or a nitrile compound) may be involved in equine ataxia and urinary incontinence seen in horses grazing sorghum hybrid pastures (Van Kampen, 1970; Wheeler and Mulcahy, 1989). There are reports of a similar condition in cattle and sheep (Bourke, 1995). After grazing grain sorghum regrowth, 54 of 330 breeding cows became ataxic and developed urinary incontinence. Wallerian degeneration of the white matter of the spinal cord, cerebellar peduncles, and cerebellum was seen histologically (McKenzie and McMicking, 1977). In humans, long-term consumption of the cyanide-containing plants tropical lima beans and cassava root is associated with well-documented conditions involving the spinal cord, optic nerves, and other lesions. A study of children in Mozambique evaluated the possible association of high cyanide and low sulfur intake in cassava-induced spastic paraparesis. The study results supported the hypothesis that the epidemic was due to the combined effects of high dietary cyanide exposure and sulfur deficiency (Cliff *et al.*, 1985). Teratogenic effects of cyanide have been demonstrated in hamsters (Frakes *et al.*, 1986).

## TREATMENT

Rapid response to intravenous antidote solution can be striking, although the opportunity to treat is rare because of the peracute nature of the poisoning. The antidote of choice in humans, dogs, and probably most other animals is 10–20 mg/kg BW of sodium nitrite in combination with sodium thiosulfate (Baskin *et al.*, 1992; Burrows and Tyrl, 2001). Ruminants can be treated with thiosulfate alone using a 30–40% solution intravenously at a dose of 25–50 g/100 kg BW (Burrows and Tyrl, 2001).

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Cyanide poisoning is an uncommon event in livestock production, but the potential for sudden economic loss to individual farmers and ranchers is significant. Veterinarians, agricultural educators, and consultants should continue to remind clientele of the risks to ruminant species associated with forages, shrubs, and trees of known cyanide potential.

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# Nitrate and nitrite accumulating plants

Steven S. Nicholson

## INTRODUCTION

Ruminants are particularly at risk of acute, fatal nitrate–nitrite poisoning. Microorganisms in the rumen reduce nitrates to nitrites and then ammonia for microbial growth. Excess intake of nitrates may cause toxic levels of nitrite to accumulate and be absorbed into the blood. Cattle graze a variety of grasses and weeds that under certain conditions, especially excessive fertilization or reduced growth rate because of drought, herbicide damage, or reduced sunlight, can accumulate levels of nitrate in the stems that may prove toxic to animals that eat a sufficient dose. Excess levels of nitrate may be present in grazed forages and weeds, in hay, or in fresh cut forage brought to the animals as greenchop. Ensiling may reduce nitrate levels by 30% or more. Sheep and rabbits can convert nitrate to toxic levels of nitrite. Goats browse leafy portions of plants and may not ingest toxic levels of nitrates in stalks and stems of forages. In horses, some bacterial reduction of nitrate to nitrite does occur in the large bowel (Bruning-Fann and Kaneene, 1993). Nitrate toxicosis in horses is rarely reported as a clinical entity (Hintz and Thompson, 1998). Monogastric species are susceptible to the toxic effects of nitrites ingested as nitrites from nonplant sources. Nitrates and nitrites are water soluble and may contaminate water sources. Forages and weeds growing in soil rich in manure waste or in holding pens are a potential source of poisoning. Nitrate fertilizers are commonly found on farms and ranches, and accidental ingestion or feed contamination does occasionally occur. Three cases of accidental acute fertilizer poisoning in cattle resulted in substantial death loss. Water hauled in tanks previously contaminated with a nitrogen-based fertilizer was the source in all cases. In two cases, analysis

of the water measured urea at 1640 and 2300 ppm and ammonia nitrogen at 640 and 750 ppm, respectively. Confirmation of urea toxicosis was made by measurement of toxic levels of ammonia nitrogen in the rumen contents (>800 ppm) and blood (>8.0 ppm). In the third case, no urea was detected in the water, but ammonia nitrogen was measured at 1670 ppm and nitrate at 1.1%. Toxic levels of nitrate were measured in ocular fluid from two animals (60 ppm). The rapid progression of signs to death with ammonium nitrate poisoning may explain the lower levels of nitrate attained in ocular fluid than observed with pure nitrate intoxications (Villar *et al.*, 2003).

## BACKGROUND

Plants take up nitrogen from the soil primarily in the form of nitrate. Nitrate accumulation in the stems and leaves of plants may be associated with high levels of nitrates, or ammonia, in the soil. Young plants are more likely to have high nitrate levels than are more mature plants. Nitrate concentrations decline considerably in all parts of sudangrasses (*Sorghum* spp.) following heading (Mizukami *et al.*, 1997). Plants growing in soil where livestock manure and urine were applied as fertilizer or where accumulations occur in holding pens may accumulate nitrates. Hungry cattle and sheep introduced to stockyards containing a dominant or pure growth of button grass (*Dactyloctenium radulans*) suffered acute nitrate–nitrite toxicity in four incidents in inland Queensland between 1993 and 2001 (McKenzie *et al.*, 2004b). The nitrate content of the button grass from within the stockyards ranged from 4.0 to 12.9% as KNO<sub>3</sub> in dry matter



and from outside the stockyards ranged from less than 0.2 to 0.4%. Three cows fed *Chenopodium album* hay died 30 min after showing ataxia, bluish-brown mucous membranes, rapid and difficult breathing, increased heart rates, tremors, and coma. Brown-colored and badly coagulated blood was the prominent necropsy finding. Slight pulmonary edema was prominent, and all visceral organs were hyperemic. The hay contained 2500 ppm nitrate–nitrogen and 11 ppm nitrite–nitrogen (Ozmen *et al.*, 2003). After harvesting corn in Nebraska, cornstalks remaining in fields had an average decrease in potassium nitrate content of only 30% in 90 days (Johnson *et al.*, 1992). Nitrate–nitrite poisoning killed four adult alpacas and induced the abortion of a full-term fetus after access to oaten hay (*Avena sativa*) containing 3.2% KNO<sub>3</sub> equivalent in dry matter. Necropsy findings were cyanosis, dark-colored blood, and pulmonary congestion and edema. Aqueous humor from two adults contained 25 ppm NO<sub>3</sub> and that from the fetus contained 10 ppm NO<sub>3</sub> (McKenzie *et al.*, 2004a).

Plant growth may be slowed, and nitrate accumulation increased, when growing in soil that contains nutrient deficiencies or excesses. For example, molybdenum is a component in enzymatic reactions of nitrate reductase in plants. Nitrate accumulation in stalks and stems may follow herbicide damage to plants or loss of leaves due to hail.

A major reason why plants accumulate nitrates is drought. During periods of drought, the growth of forages and weeds is reduced, but the roots may continue to collect and store nitrate in the stems. This is particularly true of well-fertilized sorghum hybrid (*Sorghum* spp.) and millet (*Pennisetum* spp.) forages grown for temporary summer grazing and for hay production (Clay *et al.*, 1976). Shortages of forage caused by a 2-year drought in Kansas were further exacerbated by increases of nitrate and cyanide in forages based on analyses of samples submitted to a laboratory (Pickrell *et al.*, 1991). Within a given field or pasture, forage nitrate levels may vary considerably, requiring multiple forage samples for testing to achieve accurate assessment of the risk of nitrate–nitrite intoxication. Determining the nitrate status of bales of stored hay can be a challenge because only the forage growing in a portion of a hayfield may have been affected. Bales must be labeled, sampled, and tested for nitrate content. Obtaining a representative sample requires using a hollow handheld commercial tool that cuts through to the center of the bale and recovers a core sample of approximately 1 oz. of hay. At least two samples from each bale should be collected. Investigation might reveal, for example, that one-third of the bales have nitrate levels less than 0.5%, one-third perhaps 0.5–1.0%, and the remaining portion greater than 1%.

Plants may accumulate nitrates during periods of reduced sunlight. Sunlight is needed to drive photosynthesis and the energy-dependent nitrate reductase system

in the plant. Forage or weeds growing in the shade of trees in an orchard may be subject to nitrate accumulation. Nitrate poisoning is occasionally a problem in areas of the United States where winter grazing for cattle consists of fertilized pastures of ryegrass (*Lolium multiflorum*), oats (*Avena* spp.), turnips (*Brassica rapa*), or wheat (*Triticum* spp.). During extended periods of overcast weather, perhaps several days, the nitrate content of the forage may increase to potentially toxic levels. Accumulation is more likely when temperatures are mild (>55°F) and the root systems are actively taking up nitrates. Growth slows or stops, but the roots continue uptake of nitrate, which is stored in the stems until there is adequate sunlight and growth resumes. Generally, a day or two of sunlight with temperatures above 55°F allows plant growth to continue, converting excess stored nitrate into plant protein.

To reduce trampling of forage, farm management may employ limited grazing periods, allowing hungry cattle to consume a large amount of green forage for approximately 2 h each day. This increases the risk because of the time–dose relationship that exists when excess nitrate is present and conversion of nitrate to nitrite exceeds the ability of the rumen flora to convert nitrite to ammonia. Total dietary intake of nitrate should be considered. For example, supplemental feeding of hay that has increased nitrate levels to cattle grazing forages with elevated nitrate levels increases the risk of toxicosis. Nitrate in drinking water adds to dietary intake. Nitrate concentrations in water in excess of 1000 ppm may cause nitrate poisoning in livestock.

Nitrate levels are not reduced by drying and baling as hay. High nitrate hay (>1.5% KNO<sub>3</sub>; 1.0% nitrate) fed to cattle months after baling can cause multiple deaths and abortions. Mortality can be striking, as in a case in Nebraska in which *Amaranthus/Kochia* hay with 4.9% KNO<sub>3</sub> and sudangrass with 8% KNO<sub>3</sub> were fed to 390 cattle, resulting in death of 226 and 42 abortions (Hibbs *et al.*, 1978). High nitrate summer hay fed during the winter of 1977–1978 killed cattle in Oklahoma (Haliburton and Edwards, 1978).

Rumen microorganisms can adapt to and utilize increasing levels of nitrate in the diet. The period of maximum acclimation occurs within 6 days (Allison and Reddy, 1984). Adaptation can be lost within a few days. The ability of rumen microorganisms to safely reduce nitrate and nitrite can be increased by feeding corn-based supplements to cattle (Burrows *et al.*, 1987).

Nitrate content in properly ensiled forage may be reduced by 30% or more during the ensilage process. The silage should be tested before feeding. Silage juices draining from the silo may be high in nitrates. Nitrogen dioxide (NO<sub>2</sub>) and nitrogen tetroxide (NO<sub>4</sub>) gases may be formed from oxides of nitrogen generated during anaerobic fermentation of high-nitrate forages. These pulmonary toxicants are heavy yellow-brown gases.

## PHARMACOKINETICS/ TOXICOKINETICS

Action of the rumen flora reduces nitrate to the much more toxic nitrite, which is normally converted to ammonia and further utilized by the microorganisms. Nitrite is absorbed into the blood when the intake of nitrates and the production of nitrite exceed the capacity of the rumen flora to further reduce nitrite. In some cases, preformed nitrite in hay may shorten the period from ingestion to onset of signs. Nitrates are absorbed into the blood as well but are much less toxic than nitrite. The half-life of nitrate is estimated to be 9 h in adult cattle and more than 24 h in the bovine fetus (Johnson *et al.*, 1992). The half-lives of nitrate and nitrite in the blood of sheep are 4.2 and 0.5 h, respectively (Schneider and Yeary, 1975).

## MECHANISM OF ACTION

The nitrite anion causes vasodilation and oxidizes ferrous iron ( $\text{Fe}^{2+}$ ) in hemoglobin to the ferric iron ( $\text{Fe}^{3+}$ ) state forming methemoglobin, which cannot accept molecular oxygen. As the percentage of methemoglobinemia rises, oxygen starvation to tissues increases and blood becomes chocolate brown in color. In sheep, the half-life of methemoglobinemia is approximately 1.5 h (Schneider and Yeary, 1975). Clinical signs such as exercise intolerance appear at 30–40% methemoglobinemia, with death from hypoxia likely when concentrations exceed 80% (Burrows, 1980). In nonfatal cases, a red blood cell intrinsic NADH-dependent diaphorase or reductase system gradually reduces methemoglobin to hemoglobin, which accepts molecular oxygen and can carry oxygen to tissues.

## TOXICITY

The nitrate level in edible stalks and stems of plants that is generally accepted as safe for all classes of cattle is less than 0.2–0.5% on dry weight basis. It is recommended that pregnant animals not be fed forage or hay with a nitrate content greater than 0.2% on dry weight basis. Forage nitrate higher than 1% (1.5%  $\text{KNO}_3$ ) is considered lethal. The rate of conversion of nitrate to nitrite and then to ammonia is a limiting factor in safe utilization of nitrates by ruminants. Hungry cattle are at greater risk, and intake of dry matter from hay may be faster than from grazing. The additive effect of nitrates in water and other feed sources must be considered when evaluating total dietary nitrate.

The rumen flora can safely utilize higher amounts of nitrate if sufficient dietary energy is present to promote reductive activity. Feeding corn-based supplements to cattle reduced nitrite accumulation (Burrows *et al.*, 1987). In this study, feeding of 3.2 kg of corn protected against nitrate poisoning by reducing intraruminal nitrite and blood methemoglobin ( $P < 0.05$ ).

Clinical signs of nitrate–nitrite toxicosis in cattle include weakness, cyanosis of mucous membranes, ataxia, collapse, and death. Increased respiratory rate may be noted in some animals.

Affected animals may remain standing but then collapse and die within minutes. Dead animals may be found in sternal recumbency or lying on their side. Blood is dark and may have an obvious brown color when drawn into a syringe or spread on a white cloth. At necropsy of animals dead several hours, this color may not be as apparent.

In cattle, abortions may occur in the herd 3–7 days after the acute toxicosis episode. Less oxygen is available to the fetus because of methemoglobinemia in the cow, and nitrite induces methemoglobinemia in fetal blood (Bruning-Fann and Kaneene, 1993). Bovine abortion has been reported to occur with forages containing 0.61–1% nitrate (van't Klooster *et al.*, 1990).

Differential diagnoses to consider include acute toxicoses caused by insecticides, carbohydrate overload, hypomagnesemia in lactating cattle, cyanide, blue-green algae, urea (ammonia), and potent oxidizing agents such as sodium chlorate herbicide and aniline dyes. Lesions are not diagnostic. Blood and tissues may appear brown at time of death, but this becomes less obvious as autolysis proceeds. Dark blood may suggest septicemia. Agonal hemorrhages in the epicardium may be present.

Ocular fluid is an excellent body fluid for nitrate analysis. Plasma and serum are acceptable. Ocular fluid nitrate levels are 35% lower than serum levels (Boermans, 1990). The diphenylamine blue test is widely used for testing fluids and plant tissues (Burrows, 1980; Bhikane and Singh, 1990). The diazotization test has also been used (Bhikane and Singh, 1991). Nitrate concentrations in ocular fluid of 10 ppm are indicative of excessive nitrate exposure, and concentrations greater than 20 ppm are considered diagnostic of poisoning (Burrows and Tyrl, 2001). In abortion and stillbirth situations, interpreting bovine fetal ocular fluid nitrate levels is more problematic because normal levels may approach 20 ppm in weak or stillborn calves (Johnson *et al.*, 1994). A level of 30 ppm or more and additional diagnostic information such as elevated forage nitrates may be needed to confirm nitrate abortion (Casteel and Evans, 2004).

The clinical history may suggest nitrates as a possible cause. All sources of forages, weeds, water, feed supplements, and fertilizers to which the animals had access should be determined and sampled for analysis.

## TREATMENT

Treatment is with intravenous methylene blue in a 1 or 2% aqueous solution at a rate of 1 or 2 mg/kg BW. Up to 10 mg/kg BW can be administered in severe cases. The response to intravenous treatment of a 2% solution of methylene blue at a dosage of 20 mL/100 kg BW is rapid, with reversal of the clinical signs within several minutes (Burrows and Tyrl, 2001). In severe cases, treatment at a lower dose can be repeated. Methylene blue serves as an electron carrier for an NADPH-dependent system to reduce methemoglobin to hemoglobin. Methylene blue is most effective in humans and ruminants (Burrows and Tyrl, 2001). Tissues in the treated animals are stained, and the urine becomes dark green. Treated animals should not be sold for slaughter for 180 days. Other dyes, such as tolonium chloride (tolonium blue), are effective in reducing methemoglobin to hemoglobin but have a narrow therapeutic index (Gupta *et al.*, 1992; Cudd *et al.*, 1996).

## CONCLUDING REMARKS

Safe use and storage of nitrate fertilizers is essential if accidental poisoning of livestock, especially cattle, is to be avoided. Use of liquid fertilizer tanks to deliver water to livestock is a documented hazard for nitrate or urea toxicosis. The potential for nitrate accumulation in weeds and forages intended for feeding or grazing and the risks this poses to ruminants, especially cattle, should be pointed out to farmers and ranchers by university extension and farm industry personnel. Cutter blades can be raised to reduce the amount of edible stalk in harvested forages if conditions suggest the possibility of high nitrate levels in the stalks and stems. Pre-feeding testing of potentially high-nitrate forages is especially important when environmental conditions affect growth. Feeding cattle an energy source such as corn or providing oral product containing *Propionibacterium* can increase the rate of nitrite reduction by rumen flora.

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## Toxicity of yew (*Taxus* spp.) alkaloids

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### INTRODUCTION

Yews (*Taxus* spp., Taxaceae) are evergreen plants often used for ornamental landscaping in many areas of the United States, Europe, and elsewhere throughout the world. Common varieties in the United States are English yew (*Taxus baccata*), American yew (*Taxus canadensis*), Japanese yew (*Taxus cuspidata*), and Pacific or western yew (*Taxus brevifolia*) (Kingsbury, 1964). These plants can be highly toxic and have been implicated in numerous human and animal poisonings. The poisonous taxine alkaloids have been reported to be present in the foliage, bark, and seeds of the plants but not in the fleshy scarlet aril (berry) (Bryan-Brown, 1932; Kingsbury, 1964).

### Historical background

References to yew toxicity date back more than two millennia (Bryan-Brown, 1932). In the 1st century BCE, Julius Caesar (102–44 BCE) wrote of Catuvolcus, the king of Eburones, who poisoned himself with yew “juice” (Fröhne and Pfänder, 1984). Ancient Celts often committed ritual suicides by drinking extracts from yew plants and applied the poisonous sap to the tips of their arrows during the Gaelic Wars (Foster and Duke, 1990; Hartzell, 1995). Some primitive cultures are reported to have used yew extracts as hunting and fishing aids (Watt and Breyer-Brandwijk, 1962; Hartzell, 1995). During the 18th and 19th centuries, concoctions brewed from yew leaves were documented as having been used as an abortifacient or an emmenagogue by women in Europe and India (Bryan-Brown, 1932; Watt and Breyer-Brandwijk, 1962).

### Chemical characterization

The first preparation of an amorphous, white, noncrystalline powder called “taxine” originated from an analysis of alkaloid content from yew foliage (*T. baccata* L.) in 1856 by Lucas. It was isolated in crystalline form approximately 20 years later by Marmé, a French scientist (Hilger and Brande, 1890); however, it was not until 1956 that Graf and Boeddeker discovered that taxine was a mixture of heterogeneous compounds. Further investigations using electrophoretic analysis of taxine extracts revealed two major bands, thus recognizing the presence of two major types of taxine alkaloids: taxine A and taxine B (Graf, 1956). The farthest migrating electrophoretic band was designated taxine A, which comprised approximately 1.3% of the total alkaloid extract. The other electrophoretic band, taxine B, represented approximately 30% of the total alkaloid fraction extracted from *T. baccata* L. (Graf, 1956; Graf and Bertholdt, 1957). Subsequent analyses elucidated the molecular and structural formula of taxine A as well as its basic physical and chemical properties (Graf and Bertholdt, 1957; Graf *et al.*, 1982). A structural analog of taxine A, 2-deacetyltaxine A ( $C_{33}H_{45}NO_9$ ), was isolated from the leaves of *T. baccata* in 1994 (Poupat *et al.*, 1994). The chemical and structural properties of taxine A and 2-deacetyltaxine A are given in Table 82.1 and Figure 82.1. Although the preliminary structure of taxine B was first reported in 1986 (Graf *et al.*, 1986), the molecular and structural formulas were not completely elucidated until 1991 (Ettouati *et al.*, 1991). Purified taxine fractions from various *Taxus* spp. revealed the presence of several taxine B-related compounds. Isotaxine B ( $C_{33}H_{45}NO_8$ ), a structural isomer of taxine B, is present as a major constituent in the alkaloid fractions (Poupat



TABLE 82.1 Physical and chemical properties of taxine alkaloids

Taxine	Molecular formula	Melting point (°C)	Optical rotation	UV maximum (nm)	IR maximum (cm <sup>-1</sup> )
Taxine A	C <sub>35</sub> H <sub>47</sub> NO <sub>10</sub>	204–206	[α] <sub>D</sub> <sup>20</sup> = -140°	220, 255	1780, 1250
2-Deacetyltaxine A	C <sub>33</sub> H <sub>45</sub> NO <sub>9</sub>	–	[α] <sub>D</sub> <sup>20</sup> = -106°	224, 264	1734, 1691
Taxine B	C <sub>33</sub> H <sub>45</sub> NO <sub>8</sub>	115	[α] <sub>D</sub> <sup>25</sup> = +116°	210, 277	3578, 1730
Isotaxine B	C <sub>33</sub> H <sub>45</sub> NO <sub>8</sub>	–	–	282	–

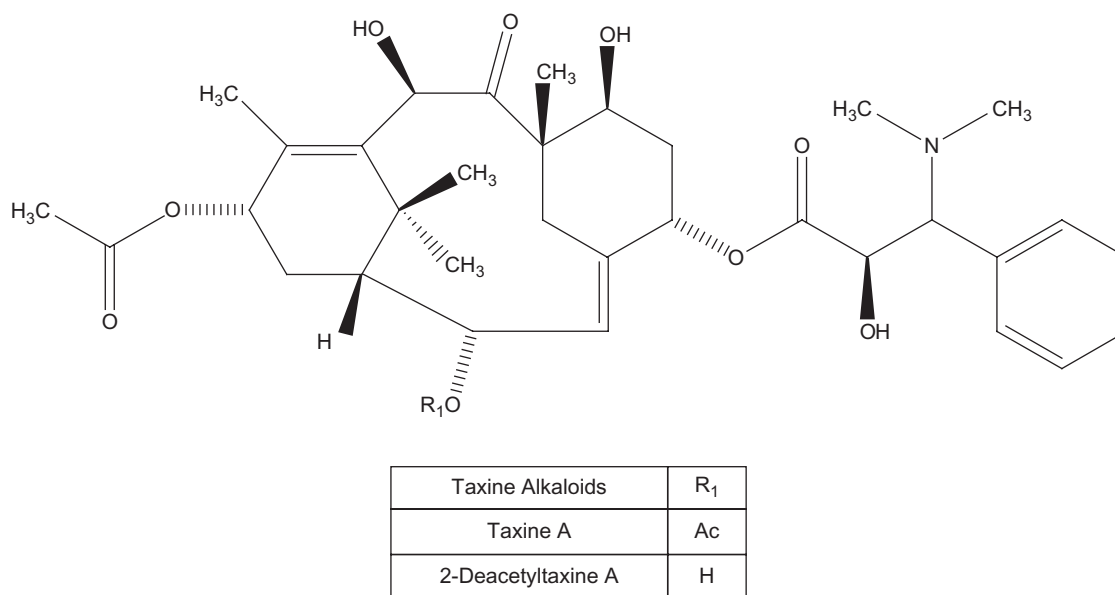


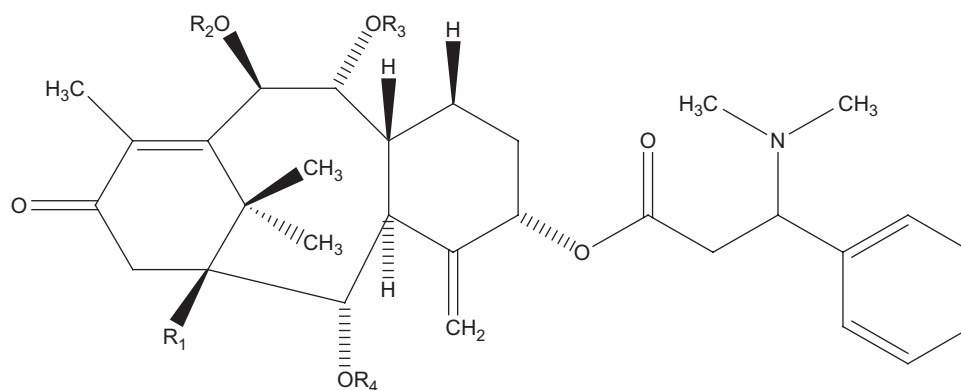
FIGURE 82.1 Structural formulas for taxines A.

*et al.*, 1994; Jenniskens *et al.*, 1996; Potier *et al.*, 1997; Adeline *et al.*, 1997). Present as minor constituents in *Taxus* spp. are 1-deoxytaxine B and 1-deoxyisotaxine B (Figure 82.2) (Jenniskens *et al.*, 1996; Potier *et al.*, 1997). The physical properties and chemical structures for taxine B and isoforms of taxine B are detailed in Table 82.1 and Figure 82.2. Other minor constituents, comprising approximately 2% of the total concentration, are the taxine B pseudoalkaloids 13-deoxo-13α-acetyloxytaxine B (C<sub>35</sub>H<sub>49</sub>NO<sub>9</sub>), 13-deoxo-13α-acetyloxy-1-deoxytaxine B (C<sub>35</sub>H<sub>49</sub>NO<sub>8</sub>), and 13-deoxo-13α-acetyloxy-1-deoxynortaxine B (C<sub>34</sub>H<sub>47</sub>NO<sub>8</sub>) (Appendino *et al.*, 1997).

## PHARMACOKINETICS/ TOXICOKINETICS

For reasons probably related to their acute toxicity and lack of pharmaceutical uses, pharmacokinetic studies on taxine alkaloids have not been well characterized. However, extensive pharmacokinetic studies have been reported for the widely used antineoplastic drugs

paclitaxel (isolated from *T. brevifolia*) and docetaxel (synthesized via a taxane precursor from *T. baccata*), which are also members of the taxane diterpenoid family. In studies of these two compounds, it has been found that they are both highly protein bound (>95%) in the serum and metabolized by hepatic P450 enzymes. One differentiating characteristic noted was that paclitaxel exhibits nonlinear kinetics at therapeutic doses, whereas the kinetics of docetaxel is linear. The linear kinetics of docetaxel is thought to be the result of metabolism by CYP 3A4 to pharmacologically inactive oxidation products that are excreted in the bile through a P-glycoprotein-dependent mechanism (Gustafson *et al.*, 2003; Baker *et al.*, 2006). Tissue distribution is extensive except for the central nervous system and testes. The elimination half-life for paclitaxel is 5–7h (two-compartment model) or 20h (three-compartment model), whereas the elimination half-life for docetaxel is 12h (two-compartment model) or 13h (three-compartment model). Liver insufficiency or co-administration of compounds that modulate P450 activity may influence the activity of these anti-neoplastic drugs and, presumably, the activity of more acutely toxic members of the family, such as taxines A and B (Brown, 2003).



Taxine Alkaloids	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
Taxine B	OH	Ac	H	H
Isotaxine B	OH	H	Ac	H
1-Deoxytaxine B	H	Ac	H	H
1-Deoxyisotaxine B	H	H	Ac	H

FIGURE 82.2 Structural formulas for taxines B.

## PHYSIOLOGY/MECHANISM OF ACTION

Due to their instability and the lack of highly purified taxines A and B for experimental use, research delving into the mechanism of action of taxines has frequently involved the use of crude extracts of taxines from yew. The earliest investigations of crude extracts of taxine alkaloids published in 1921 described cardiovascular effects. When administered by intraperitoneal or intravenous routes in rabbits and dogs, hypotension and cardiac arrest occurred in both species (Bryan-Brown, 1932). In addition, when toxicity was severe enough to result in cardiac abnormalities, it was noted that peristaltic contractions in the gastrointestinal tract ceased. Electrocardiographic analysis of isolated perfused hearts from rabbits and frogs revealed that crude taxine extracts gradually induced bradycardia, resulting in diastolic cardiac arrest. Further investigations have indicated that taxines depress atrioventricular conduction in a dose-dependent manner in isolated frog heart, having the greatest effect on ventricular rate (Smythies *et al.*, 1975; Tekol and Kameyama, 1987). In those studies, the cardiotoxic effect could not be inhibited by the administration of atropine, performing a vagotomy, or through ganglionic/adrenergic blockade (Bryan-Brown, 1932; Vohora, 1972). It was thus concluded that the hypotension induced by taxine extracts was not mediated via the sympathetic or parasympathetic nervous systems but, rather, by a direct action on myocardium (Vohora, 1972).

Significant differences in the cardiotoxicity of taxine A and taxine B have been reported (Bauereis and Steiert, 1959; Alloatti *et al.*, 1996). Through administration of taxine B either *in vivo* or *in vitro*, it was shown that taxine B is more cardiotoxic than taxine A, causing inotropic effects while eliciting marked changes in atrioventricular conduction. In isolated, perfused guinea pig hearts, a 5  $\mu\text{M}$  concentration of taxine B markedly increased atrioventricular conduction time and widening of the QRS interval, whereas a 1  $\mu\text{M}$  concentration (the lowest concentration used) significantly reduced heart rate (Alloatti *et al.*, 1996). These changes led to atrioventricular conduction blocks and complete diastolic cardiac arrest. The marked increase in QRS duration has also been reported in a human case of yew poisoning and also in intravenous administration of yew extracts to pigs (Matthew *et al.*, 1993; Ruha *et al.*, 2002). Taxine B has been shown to cause a marked reduction in the maximum rate of depolarization of the action potential in isolated papillary muscle, which thus resembles the action of class I antiarrhythmic drugs (e.g., flecainide, procainamide, and quinidine) (Bauereis and Steiert, 1959; Tekol, 1985; Alloatti *et al.*, 1996). In contrast, taxine A had a minimal effect on atrioventricular conduction time and QRS duration. Even at the highest concentration used (10  $\mu\text{M}$ ), taxine A induced only mild reductions in heart rate (Alloatti *et al.*, 1996).

More detailed investigations of the effects of taxine extracts on cardiomyocytes and axons indicated that taxines cause an increase in cytoplasmic calcium acting as calcium and sodium channel antagonists (Smythies *et al.*,

1975; Tekol, 1985; Tekol and Kameyama, 1987; Tekol and Göğüsten, 1999). In addition, isolated aorta, atrium, and jejunum from rabbits were used to compare the cardio-selectivity of taxines to verapamil, a known calcium channel antagonist. From these experiments, Tekol and Göğüsten concluded that the mechanism of action of taxines is primarily based on calcium channel antagonistic properties. Therefore, it is likely that the toxicity of taxines in animals and humans also occurs through the same mechanism.

Taxine alkaloids have minimal toxic effects on other organs. In the few studies reported, crude taxine extracts have some adverse effects on involuntary muscle but not on voluntary muscle. Uterine contractions, relaxation of the intestines, and contraction of the duodenum and ileum have been noted in experimental animals dosed with yew extracts (Bryan-Brown, 1932; Vohora, 1972). Tekol and Göğüsten (1999) reported that taxine sulfate inhibited peristaltic movement in rabbit jejunum, with a median inhibitory concentration (IC<sub>50</sub>) of  $1.86 \times 10^{-5}$  g/mL.

## TOXICITY

With the exception of the aril, all parts of the yew plant, including the seed within the aril, contain taxine alkaloids and are extremely poisonous. One study in laboratory rodents indicated that higher toxicity was found in the stems compared to the needles (leaves) (Shanker *et al.*, 2002). Although maximal concentrations occur during the winter (Watt and Breyer-Brandwijk, 1962), toxic amounts of taxines remain in the plants throughout the year and are not appreciably decreased by drying (Alden *et al.*, 1977). It has been reported that the cardio-toxic taxines A and B are relatively abundant in English

yew (*T. baccata*) and Japanese yew (*T. cuspidata*), but only minimal amounts are found in Pacific yew (*T. brevifolia*) (Tyler, 1960; Suffness, 1995; Brown, 2003).

Clinical cases resulting in poisoning are often accidental and are frequently a result of livestock or animals being unwittingly fed clippings from yew bushes. Yew intoxication has been reported in cattle (Casteel and Cook, 1985; Panter *et al.*, 1993), horses (Tiway *et al.*, 2005), sheep (Rae and Binnington, 1995) and goats (Coenen and Bahrs, 1994), dogs (Evans and Cook, 1991; Taksdal, 1994), a bear (Bacciarini *et al.*, 1999), fallow deer (Wacker, 1983), a moose (Handeland, 2008), emus (Fiedler and Perron, 1994), budgies (Shropshire *et al.*, 1992), canaries (Arai *et al.*, 1992), and monkeys (Lacasse *et al.*, 2007). It is interesting to note that yew (*T. baccata*) is often eaten by white-tailed deer and roe deer in the United States without apparent adverse effects (Weaver and Brown, 2004; Angus, 2010). This may be due, in part, to increased ruminal detoxification of the taxines present in the yew (Weaver and Brown, 2004).

Because of the difficulties in obtaining purified, stable taxines in quantities sufficient for mammalian studies, in the past, minimum lethal dose (LD<sub>min</sub>) values were assessed through the oral administration of yew leaves and branches (Watt and Breyer-Brandwijk, 1962; Clarke and Clarke, 1988). Estimating that 1 g of yew leaves contains approximately 5 mg of taxines (Smythies *et al.*, 1975; Tekol, 1985; Jenniskens *et al.*, 1996; Tekol and Göğüsten, 1999), minimal toxic doses of taxines (mg/kg body weight) in animals can be estimated and are summarized in Table 82.2. The body weights of the animals listed are average values for adult animals only (Spector, 1956). From Table 82.2, it is evident that the minimal toxic dose of taxines varies among species. Comparatively, horses are more sensitive (LD<sub>min</sub> of 1.0–2.0 mg/kg) and chickens are least sensitive (LD<sub>min</sub> of 82.5 mg/kg) to yew toxins.

TABLE 82.2 Oral lethal doses of yew leaves in animals

Animal	Estimated average body weight (kg) <sup>a</sup>	LD <sub>min</sub> of yew leaves (g)	LD <sub>min</sub> (g yew leaves/kg body weight)	Estimated LD <sub>min</sub> of taxines (mg/kg body weight)	References
Chicken	1.82	30	16.5	82.5	Clarke and Clarke (1988)
Cow	250	500	2.0	10.0	Clarke and Clarke (1988)
Dog	13	30	2.3	11.5	Clarke and Clarke (1988)
Goat	40	480	12.0	60.0	Clarke and Clarke (1988)
Horse	500	100–200	0.2–0.4	1.0–2.0	Clarke and Clarke (1988)
Pig	102	75	0.7	3.5	Clarke and Clarke (1988)
Sheep	40	100	2.5	12.5	Clarke and Clarke (1988)
Mouse	0.025	n.r.	n.r.	0.5 (s.c.) <sup>b</sup>	Tekol (1991), Tekol and Göğüsten (1999)
Rat	0.25	n.r.	n.r.	5.0 (s.c.) <sup>b</sup>	Tekol (1991), Tekol and Göğüsten (1999)
Human	80	50–100	0.6–1.3	3.0–6.5	Watt and Breyer-Brandwijk (1962)
Rabbit	2.5	1.75	0.7	3.5	Watt and Breyer-Brandwijk (1962)

n.r., data not reported.

<sup>a</sup>Estimated average body weights of adult animals in this table referenced in Spector (1956).

<sup>b</sup>Mouse and rat LD<sub>min</sub>s are based on subcutaneous (s.c.) dose.

Adverse clinical signs in animals can vary depending on the amount of yew ingested. However, in most cases of acute poisoning, animals are often found dead 24h or less after ingestion without demonstrating abnormal behavior or adverse signs of toxicity. In subacute poisonings, which have been reported infrequently, clinical signs may include ataxia, bradycardia, dyspnea, muscle tremors, recumbency, and convulsions leading to collapse and death (Evers and Link, 1972; Casteel and Cook, 1985; Veatch *et al.*, 1988; Evans and Cook, 1991; Tekol, 1991; Arai *et al.*, 1992). In cases of deliberate yew poisoning in humans, adverse symptoms of toxicity are similar to those reported in animals. Documented clinical signs in humans include dizziness, mydriasis, nausea, vomiting, diffuse abdominal pain, tachycardia (initially), severe right ventricular dilatation with biventricular dysfunction, muscle weakness, and convulsions (Blyth, 1884; Czerwek and Fischer, 1960; Fröhne and Pribilla, 1965; Schulte, 1975; Wilson *et al.*, 2001; Panzeri *et al.*, 2010). In some cases, these symptoms proceed to bradycardia, bradypnea, diastolic cardiac standstill, or death (Blyth, 1884; Fröhne and Pribilla, 1965; Schulte, 1975; Sinn and Porterfield, 1991).

## Diagnosis

Diagnosis of yew poisoning in animals is frequently based on history of exposure. Frequently, poisoning is associated with pruning bushes and then feeding the trimmings to the livestock. In some cases, yew fragments (sometimes visible only by microscopic examination) are found in the mouth, stomach content, rumen content, and/or small intestine (Frommherz *et al.*, 2006; Lacasse *et al.*, 2007; Handeland, 2008; Panzeri *et al.*, 2010). Occasionally, exposure may be indicated in the history but gross identification of intact plant material is not observed. This can be especially true in species, such as horses, that masticate their food more thoroughly. In these cases, diagnosis of taxine poisoning often requires a more detailed microscopic and/or chemical evaluation of the gastrointestinal contents (Karns, 1983; Tiwary *et al.*, 2005).

Chemical analysis of biological samples using gas chromatography/mass spectroscopy (GC/MS) or liquid chromatography/mass spectroscopy (LC/MS) has been successful in diagnosing some cases of yew poisoning in mammals (Stahr *et al.*, 1977; Kite *et al.*, 2000; Tiwary *et al.*, 2005). LC/MS has been used to detect taxine B and isotaxine B in blood (Beike *et al.*, 2003; Frommherz *et al.*, 2006) and taxines A and B from yew plant fragments (Hough *et al.*, 2010). GC/MS detection of 3,5-dimethoxyphenol, an aglycon metabolite of taxicaine, has been used as a unique marker for yew exposure in mammals. 3,5-Dimethoxyphenol has been detected in blood, urine,

bile, and gastric contents from mammals exposed to yew plants (Froldi *et al.*, 2010; Panzeri *et al.*, 2010).

At postmortem examination, there are no lesions that are pathognomonic in animals that have died due to yew toxicosis. Indeed, neither gross nor microscopic abnormalities (with the exception of large pieces of yew leaves and stems, if they are present in the gastrointestinal tract) are observed (Kingsbury, 1964; Alden *et al.*, 1977; Ogden, 1988; Rooks, 1994). An exception to this is one case of yew poisoning in a horse in which ecchymotic hemorrhages were visible grossly along the endocardial surfaces of the ventricles, and microscopically, mild multifocal necrosis of the myocardium was identified in the ventricular wall and papillary muscles of the heart (Tiwary *et al.*, 2005). In subacute poisonings, gastroenteritis may be evident; however, the inflammation is probably due to irritant oils present in the yew and not due to taxine alkaloids (Watt and Breyer-Brandwijk, 1962; Kingsbury, 1964; Evans and Cook, 1991). Rarely, other gross changes have been reported at necropsy, including moderate to severe rumenitis, superficial hemorrhages in the right ventricular myocardium and right atrium, and mild focal interstitial myocarditis (Ogden, 1988; Panter *et al.*, 1993). In cases of yew poisoning in humans, the gross anatomic observations documented include acute congestion of internal organs, cerebral edema, and hemorrhagic pulmonary edema (Beike *et al.*, 2003; Wehner and Gawatz, 2003). In these cases, histology of the myocardium revealed interstitial edema, positive staining for troponin C with depletion in areas of the left ventricle, and alveolar hemorrhagic edema.

## TREATMENT

Death is frequently the first adverse clinical sign in animals that have eaten toxic amounts of yew; therefore, opportunities to treat exposed animals are rare. However, in instances in which known ingestion has recently occurred, it is important to remove the plant material from the gastrointestinal tract and limit absorption. Rumenotomy, followed by replacement therapy with a mixture of mineral oil, electrolytes, activated charcoal, and alfalfa pellets, has been effective in treating some cases of yew poisoning in ruminants (Casteel and Cook, 1985). There is no specific antidote for taxine poisoning; however, atropine or lidocaine have been suggested to be beneficial in alleviating the cardiotoxicity (Kingsbury, 1964; Schulte, 1975). In experimental animal studies (Bryan-Brown, 1932; Vohora, 1972) and in human cases in which the cardiac responses to attempted treatment were closely monitored via electrocardiography, classic antiarrhythmic therapy proved ineffective



(Willaert *et al.*, 2002). Extreme extracorporeal life-support measures, in combination with antiarrhythmic therapy, were warranted for complete recovery in one case of yew intoxication (Panzeri *et al.*, 2010).

## CONCLUSIONS

Although advances in analytical toxicology are progressively improving detection of taxine alkaloids and other markers for diagnosis of yew poisoning, toxicoses in animals and humans still occur frequently and are usually fatal. The potent cardiotoxic effects of taxine alkaloids in the yew plant, combined with the absence of an efficacious treatment for poisoning cases, reemphasizes the need to prevent or limit exposure of animals and livestock to these toxic plants.

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## Oxalate-containing plants

Fred Reyers and Theuns W. Naudé

### INTRODUCTION

The clinical expression of oxalate poisoning is dependent on a number of factors. These interact with each other to produce a broad spectrum of clinical presentations. Oxalates play significant roles in plant metabolism and survival. Consequently, a large variety of plants contain soluble and/or insoluble forms of oxalates (Table 83.1) that may be present at different levels in different plant parts. Because oxalic acid is a strong organic dicarboxylic acid, it may be present as the acid, the acid ion (hydroxy acid ionic form), the oxalate ion (all soluble), or as a salt with a divalent cation such as calcium (Figure 83.1). Plant oxalates are present as a combination of more than one of these forms, usually with a substantial proportion of insoluble calcium oxalate crystals. The risk of poisoning by plant oxalates is largely determined by the concentration, chemical form (pH dependent), as well as the feeding behavior and gastrointestinal morphology of the exposed animal, all of which contribute to the rate and form of oxalate absorption. In monogastric animals, the ingested oxalate experiences a highly acidic environment early on, whereas in ruminants it is in a mildly acidic, almost neutral, microorganism-rich fermentation tank. In hindgut fermenters (e.g., the horse), this stage occurs after passage through the acidic gastric environment.

### OXALATE CHEMISTRY

In nature, oxalates exist almost exclusively in plant material, involving a large number of species, leading

Franceschi and Nakata (2005) to suggest the possibility that it is found in all plants. These authors published an excellent review, from which much of the information in this section is gleaned, and the reader wanting more detail is urged to obtain a copy. Oxalate is formed endogenously (in plants) through the degradation of ribulose-1,5-biphosphate (from photosynthesis) to phosphoglycine and then glycolate and its oxidation by glycolate oxidase to glyoxylate, which can be used in glycine or oxalate synthesis. A pathway from L-ascorbic acid to oxalate also exists (although not fully explicated) and appears to be a major source of oxalate in many plants (Libert and Franceschi, 1987). Because oxalic acid is a strong organic dicarboxylic acid (Figure 83.1) with two dissociation constants ( $pK_{a1}$  at 1.46 and  $pK_{a2}$  at 4.40), it can exist in several ionic forms depending on the pH of the medium (e.g., cell-sap or intestinal environment) in which it is present:

- At a pH greater than 6, it exists principally as the soluble oxalate ion (as in *Halogeton* and *Mesembryanthemum*) and, if absorbed, is able to complex with divalent cations such as plasma calcium and magnesium to sequester them as highly insoluble salts.
- At a pH between 3 and 6, oxalate exists principally as the hydrogen oxalate anion (acid oxalate ion) and forms soluble salts with sodium and ammonium in several plant species, such as grasses.
- At a pH less than 3, most of the oxalate is present in the soluble oxalic acid form, as is found in *Oxalis* and *Rumex*.

In plant cells, the insoluble calcium oxalate tends to be in the form of the monohydrate (whewellite) in most

TABLE 83.1 Plants rich in oxalates<sup>a</sup>

Family	Genus/species	Common names
Agavaceae	<i>Agave americana</i>	Tequila agave*
Aizoaceae	<i>Mesembryanthemum nodiflorum</i>	Slender ice plant
Amaranthaceae, including former Chenopodiaceae	<i>Amaranthus retroflexus</i>	Pigweed
	<i>Beta vulgaris</i>	Beetroot, mangold, sugar beet
	<i>Bassia hyssopifolia</i>	Smotherweed
	<i>Chenopodium quinoa</i>	Bitter quinoa
	<i>Chenopodium album</i>	White goosefoot
	<i>Spinacia oleracea</i>	Spinach
	<i>Halogeton glomerulatus</i>	Saltlover
	<i>Sarcobatus vermiculatus</i>	Greasewood
Araceae	<i>Dieffenbachia pictus</i>	Dumb cane*
	<i>Philodendron scandens</i>	Heartleaf*
	<i>Philodendron panduriforme</i>	Fiddle leaf*
	<i>Pinella ternata</i>	Cow dipper*
	<i>Pinella pedatisecta</i>	Green dragon*
	<i>Arisaema amurense</i>	Jack-in-the-pulpit*
	<i>Typhonium giganteum</i>	Chinese Jack-in-the-pulpit*
Cactaceae	<i>Opuntia echios</i>	Prickly pear
Euphorbiaceae	<i>Tragia ramosa</i>	Noseburn*
	<i>Tragia urticifolia</i>	Nettleleaf*
Fabaceae	<i>Glycine max</i>	Soy
Oxalidaceaea	<i>Oxalis acetosella</i>	Wood sorrel
Polygonaceae	<i>Rumex acetosa</i>	Sorrel, spinach dock
	<i>Rheum rabaarbarum</i>	Rhubarb
Poaceae	<i>Brachiaria brizanthas</i>	Beardgrass
	<i>Cenchrus ciliaris</i>	Blue buffelgrass
	<i>Cenchrus letigerus</i>	Birdwood grass
	<i>Digitaria decumbence</i>	Pangolagrass
	<i>Panicum antidotale</i>	Blue panic grass
	<i>Pennisetum clandestinum</i>	Kikuyu grass
	<i>Pennisetum purpurea</i>	Napier grass
	<i>Setaria anceps/sphacelata</i>	Golden bristlegrass
Fungi	<i>Aspergillus niger</i>	
	<i>Penicillium</i>	
	<i>Botrytis</i>	
	<i>Rhizopus</i>	
	<i>Pithomyces</i>	
	<i>Mucor</i>	

<sup>a</sup>Most have been reported to have caused poisoning.

\*Plants in which crystal raphides are a significant feature.

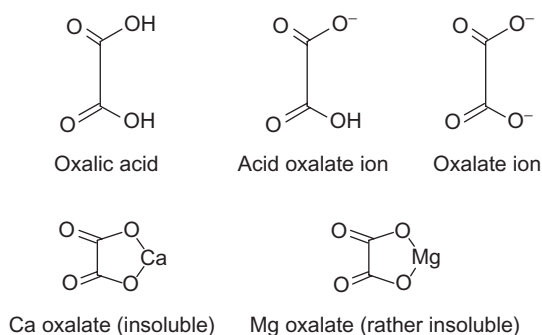


FIGURE 83.1 Oxalic acid and its salts.

cases and calcium oxalate dihydrate (weddellite) in some cases. These can be present in a number of morphological crystalline forms, the formation of which appears to be under tight genetic control. The following are the most common forms:

- Block-like rhombohedral/prismatic crystals that may be present as single or multiple crystals in each cell, often in the cell walls
- Masses of small angular crystals (crystal sand)
- Multifaceted, conglomerate crystal “druses”
- Large, elongate, rectangular crystals that can be present in bunches of needle-shaped (acicular) or single, long styloids in special intracellular structures called idioblasts. These forms are referred to as



“raphides,” some of which even have grooves and barbs. The size of these raphides can vary tremendously from microscopic to several millimeters long. They are packed together lengthwise in a gelatinous mass, and if the plant is damaged, the operculum of the idioblast is dislodged, sap of the plant or saliva causes the gelatinous mass to swell, and the needles are expelled under the pressure (Frohne and Pfänder, 1983; Cheeke, 1998). Burrows and Tyrl (2001) reported that in *Tragia* spp. (noseburns) of the Euphorbiaceae, the stinging hair of this unobtrusive creeper contains a calcium oxalate raphide up to 4 mm long that on contact penetrates the skin, allowing the highly irritant proteinaceous cell contents to enter the small wound, causing a transient pruritis.

It is believed that the presence of oxalic acid, particularly insoluble calcium oxalate, fulfills a number of functions, some of which (e.g., plant protection against herbivory by raphides and resisting parasitism (Weir *et al.*, 2006) as well as structural functions) may have been later evolutionary adaptations to the basic functions such as calcium regulation to control the very large range of calcium concentration fluxes that plants can experience as a result of poor root zone entry restriction to calcium and detoxification of potentially toxic metals such as the ubiquitous aluminum.

Plant oxalate occurs in a soluble form (oxalic acid, acid oxalate, or oxalate) and/or insoluble calcium oxalate (solubility only 6 mg/L or 6 ppm) at neutral pH. It is the soluble forms that pose the immediate threat for toxicosis. It has to be borne in mind, based on the dissociation constants, that the highly insoluble calcium oxalate (at neutral pH) becomes reasonably soluble when exposed to typical gastric content pH (<2) in monogastric animals. However, plant oxalates are seldom present as either soluble or insoluble but, rather, in varying proportions of each. For instance, grain amaranth (Gélinas and Seguin, 2007), 0.46 g/kg soluble oxalate, represents 20% of the total oxalates (i.e., 80% (1.83 g/kg) is present as insoluble crystal oxalates); taro (Savage and Dubois, 2006), 2.36 g/kg soluble oxalate, represents 58% of the total oxalates; and spinach (Brogren and Savage, 2003), 7.37 g/kg soluble oxalate, represents 77% of the total oxalates. By contrast, *Halogeton*, the cause of most reported outbreaks of oxalate toxicosis in sheep, contains only 17% soluble oxalates, but the total oxalate level is considerably higher than those of the plants mentioned previously – up to 60 g/kg (James and Butcher, 1972). Furthermore, the concentration in different parts of the plant may differ widely. For instance, kikuyu grass leaf has 15 g/kg soluble oxalate, which represents 63% of total oxalates, and stem has 7 g/kg soluble oxalate, which represents 73% of total oxalates (Marais *et al.*, 1997).

Cooked rhubarb stems owe their pleasant, acrid taste to the oxalic acid content. During World War I, a shortage of greens in Britain led to rhubarb leaves (in which the concentration of soluble oxalates is much higher than in the stems) being widely advocated as a substitute for spinach and other greens until several deaths due to renal damage were attributed to it (Ellenhorn and Barceloux, 1988).

Plants in which crystal raphides are a significant feature are indicated by asterisks in Table 83.1. They are widely grown for either their flowers or their particularly attractive foliage. They are also cultivated for the starch-rich tuberous rhizomes or as green vegetables (e.g., *Colocasia esculenta* (cocojam or taro) and *Xanthosoma* (new cocojam or okumo)). Plants intended for eating are usually cooked or baked beforehand to render them non-irritant (Purseglove, 1972).

In addition to plants, a variety of saprophytic fungi may produce oxalates and render hay toxic even without any obvious moldy appearance (Wilson and Wilson, 1961; Cheeke, 1995; Botha *et al.*, 2009).

## EATING BEHAVIOR AND GASTROINTESTINAL ANATOMY

### Monogastric carnivores

Toxicokinetic studies, in any detail, have only been published for humans. Data from these studies are probably valid as a model for other monogastric species. Studies have shown that the bioavailability from grilled spinach is  $0.75 \pm 0.48\%$  and for *Oxalis tuberosa* it is  $1.44 \pm 1.31\%$  after the first 6 h of ingestion (Albihn and Savage, 2001; Brogren and Savage, 2003). Furthermore, oxalate is absorbed by the gastric mucosa. The acidic environment favors the absorption of oxalic acid (Zhiqiang *et al.*, 2003). Monogastric carnivores have relatively tender buccal, lingual, and oropharyngeal epithelium, and they tend to chew their food. Once swallowed, the food passes into the low-pH gastric environment.

Cats are “pure carnivores” and under natural conditions will eat meat and some of the soft tissue organs of the prey species. They are “finicky” eaters with a significant olfactory component to food preference selection. Eating plants or prey gastrointestinal content is unusual and, consequently, these animals would not be expected to exhibit signs of oxalate toxicosis. It is therefore surprising that oxalate-related pathology is not uncommon. Occasionally, cats do indulge in eating plants, and they certainly do not “know” how to avoid those plants that could be toxic because there are reported incidents of lily poisoning (Langston, 2002), daffodil poisoning

(Saxon-Buri, 2004), golden dewdrop poisoning (Scanlan *et al.*, 2006), as well as *Dieffenbachia* (dumb cane) poisoning (Müller *et al.*, 1998). The latter is particularly pertinent to this discussion because this plant has a singular reputation of causing significant oropharyngeal pain in human exposure (Gardner, 1994). Thus, the contentions reported by Zaghini and Biagi (2005) that cats are such sensitive feeders that they are protected from consumption of toxic substances are open to question.

In contrast, dogs are not strict carnivores and do appear to consume a fair amount of plant material in nature (Silva and Talamoni, 2003; WenBo *et al.*, 2006). It is not clear whether this was linked to eating gastrointestinal content of their prey or simply "browsing." Even the coyote (*Canis latrans*) becomes decidedly "vegetarian" as needs arise (Hidalgo-Mihart *et al.*, 2001). Therefore, finding evidence of oxalate-induced pathology would not be totally unexpected. There are enough confirmed instances of poisoning due to plant toxins (Botha and Penrith, 2009) to expect the occasional accidental oxalate toxicosis, and this is indeed the case with *Dieffenbachia* (dumb cane) (Loretti *et al.*, 2003; Peterson *et al.*, 2009) as well as *Philodendron* (Hanna, 1986).

### Monogastric herbivores

The horse represents herbivorous animals that are both monogastric and hindgut fermenters. Horses are very finicky eaters and very unlikely to ingest anything irritant, although they are unable to select dietary ingredients to meet their nutritional needs (Lewis, 1995). Most cases of oxalate poisoning appear to develop a chronic calcium deficiency due to the trapping of calcium in the equine hindgut by oxalates. This leads to secondary nutritional hyperparathyroidism and is expressed as *Osteodystrophia fibrosa*.

In studies using guinea pigs, previously adapted to a diet high in oxalates (2%), as a model for hindgut fermenters, it has been shown that cecal bacteria could play a role in adaptation. This adaptation was negated by treating animals with gut active antibiotics and certain secondary bile salts associated with ileal diseases (Argenzio *et al.*, 1988). It is likely that if tolerance did develop, it would occur too low in the gastrointestinal tract to allow for sufficient protection from absorption.

### Ruminant herbivores

Among the three ruminant species that have been studied, cattle and sheep have been reported to develop major oxalate poisoning "outbreaks," with sheep apparently being much more commonly involved (James, 1972b). Of these three ruminant species, cattle are primarily grazers, browse eating only occasionally

when allowed the choice (Casasús *et al.*, 2009). Sheep browse less than goats (Gouri *et al.*, 2008). This implies that in terms of exposure to the Amaranthaceae/Chenopodiaceae in shrubland pastures, goats should be affected more often than sheep, which does not appear to be the case, possibly because sheep appear to actively seek diversity in their diet (Favreau *et al.*, 2010).

The lingual and oral mucosa of herbivores, especially ruminants, is highly keratinized (as a physical protection during grazing and browsing) and would likely prevent the peracute oropharyngeal reaction that is seen in dogs, cats, and humans when exposed to raphides (Gardner, 1994; Loretti *et al.*, 2003). In addition, plants that have been grazed or browsed are not chewed on first passage, entering the almost neutral rumen environment before regurgitation.

The belief that ruminants are able to distinguish toxic from nontoxic plants does not appear to be supported by evidence (Jackson *et al.*, 2010). Consequently, both sheep and cattle tend to develop severe outbreaks of toxicity when suddenly exposed to shrubs rich in soluble oxalates and especially when other, possibly preferred foods are in scarce supply. In these instances, it appears that the rumen "buffering" capacity for oxalates is exceeded and severe pathology results.

In the normal healthy rumen, some of the oxalic acid and soluble oxalates can combine with calcium and become insoluble, thereby reducing the chance of toxicity; the pH of approximately 5–7 in the rumen appears to favor the formation of calcium oxalate. This is in contrast to nonruminants with a gastric pH of 1–2 (Phillis, 1976). Ruminants have also developed an additional protective mechanism. If the rumen is adapted by gradual exposure to oxalic acid and oxalates, some microorganisms (e.g., *Oxalobacter formigenes*) which use oxalic acid as an energy source, producing carbon dioxide and formate, thrive. It is only when the microbes are unable to break down oxalic acid that toxicity results (Rekhis, 1994).

## CLINICAL TOXICOSIS CATEGORIES

Oxalates of plant origin have a very broad spectrum of toxic effects on animals and humans. Based roughly on the delay from the time of exposure to the occurrence of pathology, and accepting that there will be some degree of overlap from category to category, these effects include the following:

- 1 Peracute toxicosis, usually within minutes to hours of contact/ingestion
  - a Direct physical contact injury to the skin and oral (including tongue) and oropharyngeal mucosa by calcium oxalate crystals

It is believed that some of the cutaneous and oral/oropharyngeal pathology is caused by the additional entry of irritants, such as saponins, into the puncture wound caused by the raphide crystal (Gardner, 1994; Genillier-Foin and Avenel-Audran, 2007). If plant material containing raphides is chewed prior to being swallowed, masses of these raphides penetrate the mucous membrane of the mouth, causing alarming, severe (but usually transient) local irritation and clinical signs such as excessive salivation in people (Gardner, 1994; Chiou *et al.*, 1997; Salinas *et al.*, 2001) and even laryngospasm.

- b Direct chemical injury to the oral, oropharyngeal, and gastric/rumen mucosae
  - c Precipitation of poorly soluble oxalate crystals in the gastric and ruminal mucosal capillary blood vessels resulting in thrombosis and severe diphtheritic inflammation with invasion by opportunistic microorganisms. The vascular damage appears to precede the crystal deposition (Van Kampen and James, 1969).
- 2 Acute toxicosis, usually evident within 24 h
- a Sequestration of divalent plasma cations (Ca and Mg) leading to severe hypocalcemia and hypomagnesemia, causing muscular tetany/cramping and, in some instances, flaccid paralysis. Van Kampen and James (1969), in an experiment in which sheep were poisoned with a lethal dose of *Halogeton* (containing mainly the oxalate ion) and slaughtered sequentially every 2 h up to 8 h, suggested that Ca oxalate crystal deposition is secondary to vascular and renal cellular damage. They suggested that sequestration of intracellular Ca and Mg results in inactivation of vitally essential Ca- and Mg-dependent enzymes and consequent cell damage. This is further supported by Absan (1997), who stated that approximately half the Mg in the body is intracellular and that it is an essential cofactor in catalyzing approximately 300 enzymatic reactions, particularly those involving ATP production. In a review, James (1972b) noted that it is unlikely that hypocalcemia is the principal cause of death because the same degree of hypocalcemia due to EDTA infusion or dialysis does not result in mortality.
  - b Precipitation of poorly soluble calcium oxalate crystals in the renal tubules, causing acute nephrosis, tubular obstruction, and acute renal failure  
In humans, plasma oxalates are eliminated mainly by urinary excretion. Excretion is biphasic and peaks at 40 min and 3 h after the consumption of a warmed, commercially bought frozen spinach meal in normal healthy individuals. The initial peak, however, was absent in patients who had

undergone gastrotomy due to cancer. This again indicates the importance of the stomach in initial absorption (i.e., the first major peak). The second peak is related to delayed absorption from the intestines. When comparing oral bioavailability between the two groups, it was seen that the area under the curve was 50% greater in patients with a functional stomach. At the 3.5-h post-feeding period, these patients still had a greater extent of absorption, with a difference of 20%. The excretion of oxalates was determined to be  $0.0732 \pm 0.0294$  mg/min (Zhiqiang *et al.*, 2003).

The soluble oxalate is filtered out through the glomerulus but in the tubules binds to calcium to precipitate out as crystals on the damaged cells. Although the physical injury to the tubules may account for the nephrotoxicity, it is unlikely to be the only mechanism because the rapid transit time of 3 or 4 min in the tubules does not seem long enough for the crystals to grow to sufficient size to block tubules (Corley *et al.*, 2005; Jonassen *et al.*, 2005). James *et al.* (1971), however, noted that the original site where the crystals actually form is in the filtered fluid in the lumens of the tubules and not necessarily those associated with the oxalate-damaged cells. These crystals, however, may grow to such sizes that secondary mechanical damage is caused.

In tissue culture, it has been shown that certain chemical changes occur in the renal tubular cells once exposed to the oxalates. One of these is to produce phosphatidylserine, which would normally recruit macrophages to remove damaged cells. However, in cases of oxalate toxicity, the latter appears to promote the attachment of oxalates to the cell membrane. Other changes induced in the cell include the activation of phospholipase A<sub>2</sub>, which eventually leads to the release of bioactive lipids that alter mitochondrial function, activate caspases, and result in apoptosis (Kohjimoto *et al.*, 1999; Cao *et al.*, 2004; Jonassen *et al.*, 2005).

A third pathway may also be present because calcium oxalate induces lipid peroxidation in both renal cell cultures and rodents (Maroni *et al.*, 2005). It was concluded that the compromised cells undergo apoptosis or necrosis as a result and subsequently slough off to form the nidus over which further crystals may precipitate (Jonassen *et al.*, 2005).

- 3 Subchronic toxicosis, which develops in days to weeks, but it may take up to 3 months to develop formation of nephroliths and uroliths with associated tubular and, in some cases, urinary tract obstruction. This can lead to more chronic renal pathology, including secondary fibrosis and eventually chronic renal failure.

4 Chronic toxicosis, which may take more than 3 months or even years to develop. Nutrient deficiencies ascribed to oxalates have long been known in humans (von Unruh *et al.*, 2004). This arises from the ability of the oxalates to bind to various minerals, such as calcium, iron, and magnesium. By binding to these minerals, their bioavailability is decreased (Quinteros *et al.*, 2003). Based on this, oxalate is often classified as an “antinutrient” (Gélinas and Seguin, 2007) because it sequesters/reduces the bioavailability of important dietary constituents, leading to chronic mineral deficiencies such as nutritional secondary hyperparathyroidism in horses on certain pastures (Walthall and McKenzie, 1976). James *et al.* (1968) found that low levels of *Halogeton glomeratus* had a deleterious effect on nutrient balance in sheep. Oxalates can also decrease the absorption of calcium from other dietary sources such as milk. With long-term exposure to plant oxalates (e.g., various grasses; Table 83.1), horses will mobilize large amounts of calcium from the bony apatite, and this will eventually precipitate. In addition, although not always, or necessarily, involving oxalates of plant origin, the medical literature in general recognizes five “hyperoxalurias,” categorized as primary and secondary.

a Secondary hyperoxalurias

i Dietary hyperoxaluria – largely dealt with previously. However, “classical” peracute and acute poisoning does occur in humans and also displays some of the features discussed previously.

ii Digestive (absorptive) hyperoxaluria, which is seen in patients with various intestinal diseases and resections (especially small intestine) as well as in cystic fibrosis. In these cases, there is increased absorption of food oxalate due to a decrease in calcium chelation of the dietary oxalate and possibly an effect of bile acids on promoting oxalate absorption.

iii Idiopathic hyperoxaluria in patients dubbed “stone formers” who simply appear to absorb more oxalate without obvious reason. There is some support for a genetic underpinning cause and, possibly, this should be classified with the primary hyperoxalurias.

b Primary hyperoxalurias: In these inherited metabolic enzyme deficiency diseases, the source of the additional oxalate is usually not diet but, rather, glycine that has its origin in ammonia clearance and 4-hydroxyproline from connective tissue metabolism. In both of the primary hyperoxalurias discussed here, the “lesion” is in the inability to clear glyoxalate and glycolate into harmless glycolic acid with switching to the alternate pathway of oxalate formation. However, there is little doubt that loading such a patient with oxalate from food

would seriously aggravate the situation. In addition, it has been shown that loading idiopathic stone formers’ diets with 4-hydroxyproline aggravates the hyperoxaluria.

i Primary hyperoxaluria – type I due to a relative deficiency of alanine: glyoxalate amino transferase, a pyridoxine (vitamin B<sub>6</sub>) activated enzyme. There is experimental evidence in rodents that “pure” vitamin B<sub>6</sub> deficiency can precipitate a similar syndrome.

ii Primary hyperoxaluria – type II due to a relative deficiency of glycolate reductase-hydroxy pyruvate reductase.

In nonhuman animals, it is possible, although few cases have been thoroughly diagnostically explored, that the hyperoxaluric nephrosis in Beefmaster calves (Rhyan *et al.*, 1992), Tibetan spaniels (Jansen and Arnesen, 1990), and some cats, such as the Ragdoll and domestic short-hair (Blakemore *et al.*, 1988), represent animal equivalents of these inherited oxalate-related abnormalities in humans.

Furthermore, there is evidence that the occurrence of oxalate nephrosis in the cheetah (a pure carnivore) is related to ammonia, glycine, and hydroxyproline metabolic abnormalities (J. Mienie, personal communication).

## SPECIES-SPECIFIC TOXICITY

Based on the literature, there is little merit in supplying toxic doses. The oxalate level varies markedly between plant parts and between plants of the same species in different years and especially different cultivars (Massey, 2007).

### Sheep

Peracute toxicosis is rare, but it is occasionally seen in sheep kept in paddocks with no grazing and force-fed only, or mainly fed, raphide-rich garden prunings (Kellerman *et al.*, 2005).

In circumstances in which sheep have grazed *Halogeton* extensively, up to 1200 animals have been poisoned at a time (James, 1972a). In all cases of soluble oxalate toxicity, unadapted animals have to be abruptly exposed to large quantities of oxalate-containing plants. It is often a problem in low-rainfall areas where these are the dominant or only available plants. During feed shortage, the cut-up leaves of agave and cathodes of prickly pear (*Opuntia ficus indica*), particularly the thornless variety, are fed to stock and in excessive quantities may result in oxalate toxicosis.



According to [Shupe and James \(1967\)](#), *Halogeton* is highly toxic to sheep and produces an acute toxicosis in which animals die within 8–12h of exposure, rarely longer, and no chronic syndrome is seen. The clinical signs are dullness, lowered head, anorexia and ruminal stasis, blood-tinged frothing from the mouth, weakness, stiffness, polypnea, ataxia with jerky extensor rigidity, coma and death, with sheep lying dead where they had grazed. At necropsy, marked edema and hemorrhages are encountered in the rumen, which are caused by severe rumenitis with vascular damage. Oxalate crystals are found in the ruminal wall around the damaged vasculature. Kidneys are pale edematous and enlarged, and histopathology reveals hyalinization of the glomeruli and marked tubular dilation. Calcium oxalate crystals are encountered in the kidneys. Similarly, [Cheeke \(1998\)](#) holds that with oxalate ion poisoning (as in *Halogeton* intoxication), only acute toxicity is seen, but with acid oxalate, both acute and chronic toxicity may occur. According to [Burrows and Tyrl \(2001\)](#), the Chenopodiaceae contain both the oxalate and the acid oxalate ions as toxic principles, and they note that there are subtle differences in the syndromes caused by *H. glomeratus* and other soluble oxalate-containing plants. They maintain that *Halogeton* mainly affects sheep and, to a lesser extent, cattle. [Littledike et al. \(1976\)](#) described similar findings in acute *Halogeton* poisoning of sheep and also described the renal pathology. The kidneys were pale, swollen, and moist. On histopathology, the predominant lesion was nephrosis characterized by the widespread dilation of the convoluted and collecting tubules in the cortex. Although necrosis of the tubular epithelium is rarely seen, the tubular epithelium is generally flat and appears degenerate, particularly where the crystals impact the tubular walls. Birefringent crystals are also observed in the mucosa of the abomasums ([Panciera et al., 1990](#)).

In the case of *Mesembryanthemum nodiflorum* of Western Australia, the pH of the plant sap is 6, and the active ion is thus oxalate. The same acute syndrome as with *Halogeton* is experienced. *Oxalis* and *Rumex* intoxication are reported to cause death due to hypocalcemia. According to [Panciera et al. \(1990\)](#), acute exposure of sheep to *Rumex crispus* may cause sudden death, or animals may show severe clinical signs such as depression, salivation, coarse head tremors and stilted, ataxic gait, and recumbency due to calcium deficiency. When excited, some animals become severely ataxic, fall, and struggle to rise. Sheep affected by the subchronic form of the disease develop azotemia and hypocalcemia.

In experimental *Halogeton* poisoning, [Van Kampen and James \(1969\)](#) noted that the deposition of crystals appeared to be secondary to cellular damage and not vice versa. Some sheep died peracutely with

insignificant morphologic kidney lesions, and even in sheep with severe kidney damage, death occurred too rapidly to be attributed to renal dysfunction. Sheep with a bilateral nephrectomy live longer than those with acute oxalate intoxication. These authors maintained that in *Halogeton* poisoning, the serum calcium is within the range in which tetany should occur but it does not. Signs preceding death are in fact extreme weakness and flaccidity of all skeletal muscles.

A slightly different syndrome was reported by [James \(1972a,b\)](#) with *Bassia actinophylla* of the Chenopodiaceae, in which on an oxalate basis, the lethal dose for sheep is approximately one-half that of *Halogeton*. The signs resembled those of *Halogeton*, but there was a greater tendency to develop tetany and incoordination, and less than half the lethal amount fed per day resulted in a cumulative effect and mortality. Compared to *Halogeton*, this plant was higher in potassium and lower in sodium, suggesting that the difference was due to the cations. *Setaria sphacelata*, with an acidic sap, is believed to contain ammonium oxalate ([James et al., 1971](#)). In Australia, it causes the typical acute syndrome in sheep due to the oxalate ion ([James et al., 1971](#)).

The effect of acutely toxic doses of the different cations of oxalate fed daily by rumen fistula to unadapted sheep was reported by [James \(1972a,b\)](#). In a preliminary trial prior to testing the adaptation of sheep to *Halogeton*, he found that sodium oxalate at approximately 25–45g oxalic acid equivalent on the first day and 40–74g on the last day killed three sheep 3–6 days after they had been off feed all the time. Diarrhea and severe edema and hyperemia of the rumen wall were evident. Two sheep dosed with potassium oxalate at approximately 25 and 42g on the first day and 56 and 67g oxalic acid equivalent on the last day took 6 days to die, and they were only slightly off feed for the last 3 days. There was less diarrhea, and the effect on the rumen wall was less severe. In contrast, magnesium oxalate at 70.6g oxalic acid equivalent, dosed to one sheep for 1 day, caused only diarrhea.

Chronic toxicity has also been reported to occur in sheep. Animals demonstrated clinical signs over 2–12 months, with the principal signs being anemia and loss of condition and appetite. On autopsy, the kidneys were half the normal size and weight, and they were pale and mottled. In animals that were anemic, the hearts were enlarged ([McIntosh, 1972](#)).

In South Africa, mesems of the Aizoaceae are widely distributed in the western, semidesert areas, and although they are known to contain toxic quantities of oxalates ([Steyn, 1934](#)), they are so widely utilized that stock (especially sheep and goats) consume them in small quantities as part of their daily diet and frank toxicity, as in Western Australia, is not seen.

## Cattle

Burrows and Tyrl (2001) reported that the clinical signs in cattle are similar to those in sheep but less acute. Signs include incoordination, apprehension, belligerence, excess salivation, recumbency, coma, bloat, cyanosis, and death. Many of the clinical signs are associated with hypocalcemia (serum calcium as low as 1.4 mg/dL (0.35 mmol/L)), during which time blood magnesium and phosphorus may double. Subacute locomotor disturbances are mainly evident when animals are forced to move, and this begins with the forelimbs. In calves, however, more severe signs of hypersensitivity to stimulation and seizures are possible.

Lincoln and Black (1980) reported acute *H. glomeratus* poisoning in 16 of 680 range cattle during and following a trail drive. Signs of toxicosis included ataxia, recumbency, coma, and death. Histopathology revealed abundant, refractile calcium oxalate crystals in renal tubules. They suggested that although the plant is generally unpalatable for cattle, the toxicosis was precipitated by a preceding period of food deprivation. In cattle, oxalate toxicity induces catarrhal abomasitis, enteritis, pale edematous kidneys, and congested lungs (Dickie *et al.*, 1978). According to Walthall and McKenzie (1976), affected cattle on oxalate-containing pasture grasses (probably ammonium oxalate), much like sheep, tend to show signs of depression, anorexia, and diarrhea. On autopsy, the kidneys are pale and firm. On cut surface, the renal medulla is thin, and the calyces are dilated (confirmed histopathologically). Cortical and medullary tubules are distended with crystalline casts. The crystals displayed typical birefringence under polarized light and were specifically identified with Pizzolato's technique (peroxide-silver staining). In one incident, reported by Kellerman *et al.* (2005), 4 of 40 nursing beef cows developed acute flaccid paralysis 24 h after introduction to a harvested wheat land heavily infested with *Chenopodium album* (white goosefoot). The paralysis was so marked that the investigating veterinarian suspected botulism. The plants contained 16% oxalate (total) on dry matter basis.

Gopal *et al.* (1978) suggested that oxalates may also play a role in abortions and possibly teratogenicity. However, in their sequential sacrificing experiments (*vide supra*), Shupe and James (1967) found no crystalline deposits in the tissues of fetuses of ewes that had been pregnant during exposure to *Halogeton*.

Why ruminants do not "classically" develop chronic hypocalcemia and secondary nutritional hyperparathyroidism, as horses do, is not immediately obvious. Marias *et al.* (1997) estimate that at least 95% of the calcium in kikuyu grass is bound to oxalate and would be poorly available to ruminants. It is possible that when this "sequestered" calcium passes through the

abomasum, a sufficient amount is solubilized to cope with the dietary needs – calcium oxalate being three times more soluble (although still relatively insoluble) at gastric pH than at neutral pH.

## Horses

Both acute and chronic toxicity can occur in horses. However, chronic toxicosis is by far the more common naturally occurring form and is usually due to grazing pasture that is relatively high in oxalates, especially *Setaria* spp. (Jones and Ford, 1972).

Chronic poisoning following 2–8 months of exposure to oxalate-containing grasses resulted in nutritional secondary hyperparathyroidism, causing *Osteodystrophia fibrosa*. Clinical signs observed in these animals were lameness, ill thrift (harsh coats and loss of condition), and, in some animals, swelling of the osseous structure of the head. Mildly affected horses showed a decreased ability to work, whereas the more severely affected animals became cachectic and even died. The swelling to the head was bilateral and involved the nasal bones or the maxillae. Histopathologically, a decrease in osseous tissue could be confirmed. Fibrous tissue surrounded small fragments of old bone in which sites of osteoclastic activity were detected (Walthall and McKenzie, 1976). Although, in general, *Panicum* spp. grasses have considerably lower oxalate content than *Setaria* and *Cenchrus*, there may be local conditions leading to sufficiently high oxalate levels in this species to cause equine *Osteodystrophia fibrosa* (Curcio *et al.*, 2010).

For experimental acute toxicity to occur, animals need to be exposed to a very high dose of 454 g of sodium, potassium, or ammonium oxalate. This toxicity is characterized by hypocalcemia and will result in muscle rigidity and a stiff gait (Laan *et al.*, 2000). It is interesting, however, that there are no described instances of horses being poisoned by accidental ingestion of high-oxalate plants. Certain grasses can develop high enough oxalate levels to inflict acute toxicity in horses, with the attendant hypocalcemia and oxalate nephrosis. Nonfatal toxicity occurs when animals are exposed to 200 g of oxalic acid/day for 8 days (Andrews, 1971). In foals, exposure to *Rumex* spp. resulted in signs of hypocalcemia (Laan *et al.*, 2000).

Renal failure, as a subchronic and chronic presentation, has also been reported. The clinical signs are anorexia and gradual weight loss. Although the kidney appeared normal, on histopathology the renal cortex contained dilated tubules lined with flattened or degenerated epithelial cells. Tubular structures were displaced by fibrous tissue, whereas the glomeruli had undergone various degrees of degeneration. Crystals were present in the tubules, particularly the proximal convoluted

tubule, and were yellowish brown and aggregated into rosettes having radial symmetry. The crystals were anisotropic under polarized light (Andrews, 1971).

### Other hindgut fermenters

A 12-month-old black rhinoceros calf (*Diceros bicornis*) was reported by Wood *et al.* (1997) to have chewed half a leaf of an ornamental *Xanthosoma mafaffa* (elephant's ear or new cocoyam). The calf promptly spat it out, accompanied by severe salivation, flicking her tongue, and rubbing her mouth in mud for an extended period. She tried to browse after a while but spat out the food. These signs lasted for 3 or 4 h, and then she made an uneventful recovery.

Pigs have a strongly acidic gastric pH (like horses), but only one incident of oxalate poisoning is on record, following the consumption of beet. However, there is uncertainty because beet also contains nitrates and a "brown discoloration of the blood" was reported (Rupprecht, 1932). One would expect more reports of intoxication in this exceptionally widely farmed commodity and species (especially where they are farmed free-range) if oxalosis was indeed a problem in swine. Baxter (1956) described fodder beet poisoning in pigs on the basis that Gregor reported finding oxalic acid in toxic amounts in sugar beet tops (Gregor, 1953), but the signs were not typical. Beasley (1999) stated that convulsions may also occur in oxalate toxicosis in pigs.

### Dogs and cats

The predominant form of acute oxalate-associated pathology in dogs is due to consumption of ethylene glycol (antifreeze), discussed elsewhere in this book (Chapter 60). However, accidental ingestion of some high-oxalate plant material (*Dieffenbachia* and *Philodendron*) has been described, and the clinical presentation is typically of the peracute type with severe erosive/ulcerative glossitis, stomatitis, and oropharyngeal edema leading to dyspnea and airway obstruction in dogs (Loretto *et al.*, 2003; Peterson *et al.*, 2009) and extensive gastric ulceration and hematemesis in cats (Müller *et al.*, 1998).

#### *A growing problem: canine and feline oxalate urolithiasis. Is it chronic oxalate toxicosis?*

Despite the occasional reports of canine and feline dietary "indiscretion" leading to peracute and acute toxicoses, it would be reasonable to assume that the carnivores, in general, would have a very low intake of oxalates (assuming that dietary oxalate is invariably of plant origin). Consequently, oxalate urolithiasis

(the subchronic form of oxalate poisoning) should be of minor significance. However, based on reviews of a number of substantial uroliths databases of the University of Minnesota (451,891 stones, 1981–2007) (Osborne *et al.*, 2009), University of California at Davis (25,499 stones, 1985–2006) (Low *et al.*, 2010), and the University of Guelph (>50,000 stones, 1998–2008), we have been made aware of a significant and growing problem of feline and canine oxalate urolithiasis. In dogs, oxalate urolithiasis has increased from 5% of all stones examined in 1981 to 50% in 2008. In cats, the 1981 starting point was 2% and it peaked at 55% in 2002, declining to just under 50% in 2008. By contrast, cattle (1.8%) and sheep (0.9%), the known consumers of plant oxalates, appear to have a very low oxalate uroliths incidence (similar to the 1981 cat and dog prevalence) (Osborne *et al.*, 2009).

Two substantial epidemiologic studies evaluating dietary factors/contributions were conducted on cats (Lekcharoensuk *et al.*, 2001) and dogs (Lekcharoensuk *et al.*, 2000). Although these studies clearly identified a number of diet-related factors, it seems counterintuitive that the source of the oxalate was not considered an issue. In the pet food industry, corn gluten meal has been a commonly used protein source in combination with soy products (de Godoy *et al.*, 2009). Bran has a relatively high oxalate content, and soy has a high oxalate content (Massey *et al.*, 2001). From the medical literature, it appears that human "stone formers" are advised to adjust their diets to reduce oxalate intake (Grentz and Massey, 2002).

This topic was reviewed by Dijkster *et al.* (2011). It appears that in dogs, the little evidence that exists regarding the contribution of dietary oxalate to oxalate urolithiasis points to a complex, inconsistent interaction between food oxalate and calcium, with a high food calcium:oxalate ratio being protective. In cats, however, there are no such studies, and the authors suggest that the situation may be similar to that in dogs. In addition, the authors propose that in cats, endogenous production of oxalate from glyoxylate (from carbohydrate/amino acid metabolism) may play an important role but emphasize that the relative contribution of dietary oxalate is unknown.

### Poultry

Commercially produced chickens are unlikely to be exposed to oxalate poisoning. However, in an investigation of the contribution of a mixture of miserotoxin, nitrates, and oxalate involved in a particular syndrome, Williams and Olsen (1992) established the LD<sub>50</sub> of sodium oxalate for 1-week-old chicks to be 984 mg/kg.

## TREATMENT

Animals suffering from hypocalcemia have been treated with intravenous calcium (Laan *et al.*, 2000; Kellerman *et al.*, 2005). Following the development of acute clinical signs, oral dosing with lime water/milk or calcium lactate followed later by an emetic (in appropriate species) may be helpful. The rationale is to bind the unabsorbed oxalates to calcium in the gastrointestinal tract and remove it from the system. Activated charcoal may also be used. In animals already convulsing, the use of emetics (where applicable) is not recommended. In these animals, the plasma calcium levels should be monitored and treatment with calcium instituted. Calcium borogluconate is the standard treatment for most oxalate plant intoxications (Kellerman *et al.*, 2005), but in *Halogeton* poisoning in sheep, it may delay death but not necessarily result in survival. Van Kampen and James (1969) postulated that intracellular inactivation of Ca- and Mg-dependent enzyme systems may be significant in causing death.

## PROPHYLAXIS/ADAPTATION

In ruminants, dicalcium phosphate has been recommended to reduce the likelihood of *Halogeton* toxicity when mixed into the ration at a ratio of 1:3 with salt. Alternatively, 5% dicalcium phosphate-containing alfalfa pellets have been fed at a rate of 100 g to 2 kg (3–5 lb) per sheep per day (Beasley, 1999). The purpose of this is to have all the soluble oxalates precipitated in the rumen.

It has been shown that rumen microorganisms such as *O. formigenes* can metabolize oxalates. However, their numbers in the rumen appear to be dependent on the period of exposure. A study in goats indicated that artificial exposure of ruminants to oxalates can create adequate microorganism adaptation to allow an animal to cope on high oxalate-containing plants. The same may be achieved by gradually exposing animals artificially to the plants that contain oxalates (Frutos *et al.*, 1998). With *Halogeton*, James (1972a,b) experimentally determined that sheep were 5–10 times more resistant after 8–25 days of feeding, and they advised an adaptation period of 10 days prior to known field exposure that would increase the lethal dose by approximately 30%.

## CONCLUSIONS

The toxicity of oxalate-containing plants is difficult to assess and predict on the basis of the oxalate

concentration in the plant material alone. A number of additional factors interact and play a significant role in the eventual expression of a broad spectrum of clinical presentations. These are the pH of the plant material and the ionic form of the oxalate (which largely determine the solubility and availability), the feeding habits of the exposed animals, the availability of alternate/additional food sources, the total dose consumed, gastrointestinal conformation/anatomy (monogastric, ruminant, and hindgut fermenter), dietary divalent cation content, and endogenous production of oxalate from carbohydrate and amino acid metabolism. There is a paucity of published data on the role of these factors and their interactions in the various animal species. Thus, there is an urgent need for well-designed investigations to facilitate evidence-based diagnostic approaches to cases of suspected poisoning and provide safe food for domesticated animals as well as wild animals in captivity.

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# Mushroom toxins

Birgit Puschner

## INTRODUCTION

Management of mushroom poisonings of humans and animals can demand extensive effort from clinicians and toxicologists and often involves emotion and publicity. The public expects the toxicology profession to provide guidance and a coherent approach regarding these cases. Although it is estimated that very few species are lethal, it is not clear how many of the mushrooms worldwide contain potentially toxic compounds. New species are being discovered continuously, and for many species, toxicity data are unavailable. It is very difficult to establish a confirmed diagnosis of mushroom poisoning in animals; thus, clinical reports of mushroom poisoning are uncommon. In fact, because animals are at much greater risk of exposure to toxic mushrooms than are humans, mushroom poisonings in animals are most likely underreported.

## BACKGROUND

The reported frequency of mushroom poisonings in veterinary medicine is low because routine diagnostic methods to confirm exposure are lacking. Most cases are diagnosed by positive identification of the suspect mushroom along with the occurrence of consistent clinical signs and clinicopathological changes. Animals are often left unattended, and a history of mushroom ingestion is not available. An estimated 200–300 calls related to mushroom ingestions have been registered by U.S. animal poison control centers each year. California accounts for more than 10% of these cases, the highest

among all states. California also leads the United States in the number of reported cases of mushroom ingestions in humans; 894 cases were reported in 2008. Many factors influence the toxicity of mushroom toxins, such as genus and species of mushroom, geographic location where the mushroom is grown, preparation of mushroom prior to ingestion, and the individual's susceptibility. Although not inclusive of all mushroom toxins, this chapter is organized by the various types of toxins, providing detailed information on their toxic mechanisms, toxicokinetics, and diagnostic and therapeutic approaches, with a focus on veterinary medicine.

## HEPATOTOXIC CYCLOPEPTIDES

Worldwide, most fatalities are caused by exposure to cyclopeptides. Three genera – *Amanita*, *Galerina*, and *Lepiota* (Lincoff and Mitchel, 1977a) – are known to contain hepatotoxic cyclopeptides, with *Amanita phalloides*, the ubiquitous death cap or death angel, and *Galerina sulphurescens* being considered the most toxic worldwide. *Amanita phalloides* (Figure 84.1) is found throughout North America, commonly in association with oaks, birch, and pine, and it is the species most frequently resulting in fatalities in humans (Mitchel, 1980; Barbato, 1993) and probably dogs. It can also be found in open pastures. *Amanita phalloides* is particularly common in the San Francisco Bay area, the Pacific Northwest, and the northeast, and it is most abundant in warm, wet years. The large fruiting bodies appear in the late summer and fall, and they have several characteristics: a smooth, yellowish-green to yellowish-brown cap; white gills; a





FIGURE 84.1 *Amanita phalloides* (courtesy of Dr. R. Michael Davis, University of California at Davis).



FIGURE 84.2 *Amanita ocreata* (courtesy of Dr. R. Michael Davis, University of California at Davis).

white ring around the upper part of the stem (veil); and a white cuplike structure (volva) around the base of the stem. *Amanita ocreata* (Figure 84.2) is commonly known as western North American destroying angel and grows from Baja California, Mexico, along the Pacific Coast to Washington. *Amanita ocreata* is most commonly found in sandy soils under oak or pine and has caused fatalities in dogs (B. Puschner, unpublished data). The fruiting bodies are usually found in late winter and spring. *Amanita ocreata* has a white or cream-colored cap; white, short gills; a white stem with a white, thin, broken, partial veil (annulus); and a white, thin volva. *Amanita bisporigera*, *Galerina autumnalis*, and *Lepiota josserandii* have also been attributed to animal and human deaths in North America (Beug, 2009). In eastern Europe, *Galerina sulphurea* is considered the species most commonly associated with human fatalities, followed by *A. phalloides* (Klan, 1993). There are three groups of cyclopeptides – the amatoxins, phallotoxins, and virotoxins. Amatoxins are bicyclic octapeptides and include the amanitins ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\epsilon$ -amanitins), amanin, amanullin, and proamanullin (Vetter, 1998). Severe poisonings and lethality are mainly attributable to the amanitins. The bicyclic heptapeptides phallotoxins were once thought to be the cause of gastrointestinal clinical signs; however, they are no longer believed to exert any acute toxicity. Although research is limited, bicyclic heptapeptides virotoxins are not considered to have toxic effects after oral exposure. Therefore, phallotoxins and virotoxins are not discussed further.

### Pharmacokinetics/toxicokinetics

Exact data on the bioavailability for amanitins are lacking, although there are known species differences

(Faulstich and Fauser, 1980). The bioavailability of amanitins appears to be much greater in humans than in rodents, dogs, and rabbits. Within animal species, the absorption rate of amanitins is estimated to be much greater in dogs than in mice and rabbits, and rats appear resistant to the toxic effects of amanitins.  $\alpha$ -Amanitin is taken up by cells in the gastrointestinal tract, where the first damaging effects are seen (Gundala et al., 2004). Following systemic absorption,  $\alpha$ -amanitin is taken up by hepatocytes via OATP1B3, an organic anion-transporting polypeptide (Letschert et al., 2006). The  $\alpha$ -amanitin has a low volume of distribution. Renal clearance is high and similar to the creatinine clearance (Faulstich et al., 1985). Following intravenous (i.v.) administration in dogs, it was shown that plasma half-life of amanitins is short, ranging from 25 to 50 min, and that amanitins are not detectable in plasma after 4–6 h. There is no known metabolism or plasma protein binding of the  $\alpha$ -amanitin. Between 80 and 90% of the administered dose of amanitins is eliminated in urine, and up to 7% is eliminated in bile (Faulstich et al., 1985). After oral ingestion of *A. phalloides* in humans,  $\alpha$ - and  $\beta$ -amanitins were detected in plasma up to 36 h after ingestion and in urine up to 72 h post-exposure (Jaeger et al., 1993). This may partly be due to slow intestinal absorption, enterohepatic circulation, and reduced renal elimination resulting from nephrotoxicity. Plasma and urine amanitin concentrations do not seem to correlate with the clinical severity or outcome.

Amanitins can be detected in serum and urine well before any clinical sign of poisoning, whereas routine laboratory tests such as complete blood count and serum chemistry profiles are unremarkable until liver or kidney damage has occurred. Early recognition of exposure is critical because survival rates are greatly improved with



timely therapeutic intervention. Amanitin concentrations in kidneys and livers of people ingesting *A. phalloides* have been detected up to 22 days post-ingestion. The kidneys contain higher concentrations than the liver, indicating that toxins are bound to renal tissue.

## Mechanism of action

Amanitins are of greatest significance because, unlike phalloidins, they are heat stable and are not degraded by the acid environment of the stomach or by freezing (Himmelmann *et al.*, 2001). Therefore, amanitins are toxic by ingestion, whereas phalloidins have only been shown to be toxic when experimentally administered by parenteral routes. Amanitins exert their toxicity by inhibiting nuclear RNA polymerase II (Lindell *et al.*, 1970; Wieland, 1983). The decrease in mRNA and associated decrease in protein synthesis result in hepatocyte necrosis. Cells with a high metabolic rate, such as hepatocytes, crypt cells, and proximal convoluted tubules of the kidneys are most commonly affected. Although this mechanism is well established, research has confirmed additional cellular effects that contribute to pathogenesis. In mice and cultured dog hepatocytes, apoptosis contributed to amanitin-induced liver failure (Leist *et al.*, 1997; Magdalan *et al.*, 2010b), and in pancreatic rat islets  $\alpha$ -amanitin resulted in a dose-dependent insulin-releasing and a  $\beta$ -cytotoxic effect (De Carlo *et al.*, 2003). Acute tubular necrosis in the kidneys is believed to be a result of reabsorption of amanitins by renal tubules after glomerular filtration.

The clinical course is divided into four phases, with the initial phase being the latency period of approximately 6–12h. The second phase is characterized by severe gastrointestinal signs, such as nausea, vomiting, bloody diarrhea, and severe abdominal pain. In dogs, the onset of clinical signs is generally 6–24h after mushroom ingestion. Beagles experimentally given an oral sublethal dose of amanitins developed vomiting and diarrhea at 16h. Gastrointestinal signs improved after 60h (Vogel *et al.*, 1984). The gastroenteric phase is often followed by a lag period of several hours to a few days during which the human or animal will appear to have recovered. During this third phase, close monitoring of liver and kidney function is essential in order to prevent misdiagnosis. The final stage begins approximately 36–84h after exposure to amanitins. In this stage, fulminant hepatic failure with subsequent coagulation disorders, encephalopathy, and renal failure may occur. In dogs, significant elevations in serum of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, and bilirubin are commonly observed (Vogel *et al.*, 1984; Kallet *et al.*, 1988). In humans, a combination

of the prothrombin index along with serum creatinine concentrations determined between 3 and 10 days after ingestion was most useful as a predictor of death (Ganzert *et al.*, 2005). Although no controlled study exists in dogs, prothrombin (PT) and partial thromboplastin (PTT) may provide critical information for case assessment (Tegzes and Puschner, 2002). Clinical signs of renal failure include polyuria, polydipsia, vomiting, and anorexia. Severe hypoglycemia can occur in dogs after the gastrointestinal phase, and it is associated with the breakdown of liver glycogen (Puschner *et al.*, 2007). In a study in which dogs were given lethal doses of amanitin toxins or pieces of *A. phalloides*, 50% of dogs died from hypoglycemia 1 or 2 days after exposure (Faulstich and Fauser, 1980). In clinical cases, dogs must be monitored closely for hypoglycemia, and treatment may be necessary. Clinical signs of hypoglycemia in dogs and cats include seizures, coma, and death. Finally, it is important to note that not all cases present with the classic four stages. In cases of large exposure to amanitins, or ingestion by puppies, the animal may die within 24h (Cole, 1993).

## Toxicity

Amanitins are extremely toxic, and poisonings have been confirmed in dogs, horses (Beug, 2009), and cattle (B. Puschner, unpublished data). The i.v. LD<sub>50</sub> of  $\alpha$ -amanitin in dogs is 0.1mg/kg body weight (Faulstich *et al.*, 1985). Based on an oral dosing study in dogs, the oral LD<sub>50</sub> for methyl- $\gamma$ -amanitin was estimated to be 0.5mg/kg body weight. In mice and rats, the i.v. LD<sub>50</sub> of  $\alpha$ -amanitin is 0.35 and 3 or 4mg/kg body weight, respectively, illustrating significant species differences. Guinea pigs and rabbits are considered to be approximately equally sensitive as dogs to amanitins, with i.v. LD<sub>50</sub>s of  $\alpha$ -amanitin of 0.1 and 0.2mg/kg, respectively. In humans, the estimated oral LD<sub>50</sub> of  $\alpha$ -amanitin is 0.1mg/kg body weight. Considering the average concentration of amanitins per mushroom, one *A. phalloides* has the potential to kill a dog or horse.

## Treatment

No specific antidote for amanitins exists, and thus there is wide variability in treatment and overall response rate. Prompt measures, including decontamination and supportive care, are required to improve prognosis. Even with supportive measures, the reported mortality rate from *Amanita* poisoning in humans is 15–20%, and it is often higher in children (Enjalbert *et al.*, 2002). In humans with amanitin poisoning, silibinin, penicillin, and *N*-acetylcysteine are most commonly recommended,

although clinical efficacy data are ambiguous. Similar treatment approaches have been used with variable success rates in dogs suffering from amanitin poisoning (Tegzes and Puschner, 2002).

Silibinin and penicillin reduce the uptake of amanitins into hepatocytes. Silibinin (also known as silybin) is the main component of silymarin, which is extracted from the common milk thistle (*Silybum marianum*). Silibinin is also a free radical scavenger, and it has immunostimulatory and iron binding properties (Mayer *et al.*, 2005). In Europe, a silibinin-contained product (Legalon-Sil) is a well-established and approved treatment for amanitin poisonings in humans. Treatment consists of a 5mg/kg loading dose i.v. followed by 20mg/kg/day via continuous infusion (Karlson-Stiber and Persson, 2003) until coagulopathy is no longer present and liver function tests have returned significantly toward normal. In 2009, an open clinical trial was sanctioned by the U.S. Food and Drug Administration and is currently being conducted in northern California (<http://clinicaltrials.gov/ct2/show/NCT00915681>). In dogs, silibinin was shown to be effective when given twice at a dose of 50mg/kg i.v. 5 and 24h after exposure to *A. phalloides* (Vogel *et al.*, 1984). Dosed dogs had better indices of liver function as assessed by serum elevations of AST, ALT, bilirubin, and prolonged prothrombin time. On histopathology, no hepatic lesions were found. Side effects of silibinin administration are rare but include anaphylactic reactions, mild laxative effects, and interactions with certain phase I and phase II metabolism enzymes (Venkataramanan *et al.*, 2000). Oral administration of milk thistle preparations is not recommended because they are poorly absorbed.

Penicillin G was shown to protect against amanitin-induced cell damage in cultured human hepatocytes (Magdalan *et al.*, 2010a). Mice given 1000mg/kg of penicillin G intraperitoneally 8h after exposure to a lethal dose 95 (LD<sub>95</sub>) of amanitin had less morbidity and mortality than did control mice (Floersheim, 1972). In dogs, i.v. administration of 1000mg/kg of penicillin G at 5h post *A. phalloides* exposure was considered an effective treatment (Floersheim, 1978).

The benefits of several antioxidants in amanitin intoxications have been evaluated in humans. Most information is available for *N*-acetylcysteine (NAC), which was shown to be as effective as silibinin in reducing mortality (Enjalbert *et al.*, 2002) and protecting against cell damage (Magdalan *et al.*, 2010a). In mice, NAC administration was not effective (Schneider *et al.*, 1992). Although efficacy data of NAC administration in dogs with amanitin poisoning are lacking, there is no reason not to include the glutathione precursor in the treatment regimen. Ascorbic acid may also be of benefit when managing amanitin poisoning in dogs, but specific data are not available. In contrast, cimetidine, thiocetic acid, and

steroids are no longer recommended because of poor clinical efficacy.

With the identification of OATP1B3 as the primary hepatic uptake transporter for amatoxins in humans, high-affinity substrates and inhibitors of OATP1B3 provide excellent candidates for antidotes (Letschert *et al.*, 2006). Silibinin and penicillin G are in this category, but rifampicin, cyclosporine A, and montelukast must be further evaluated because they may be superior in preventing amanitin uptake into hepatocytes.

Hemodialysis, hemoperfusion, activated charcoal, plasmapheresis, forced diuresis, and nasoduodenal suctioning have been used to treat amanitin poisonings. Controversy remains with regard to the efficacy of decontamination procedures because specific efficacy data do not exist. However, there is general agreement that activated charcoal is beneficial. In dogs, multidose activated charcoal is given every 2–6h until 2 or 3 days post-ingestion. Close monitoring, fluid replacement, and supportive care are essential for treatment. Intravenous fluids, correction of hypoglycemia and electrolyte imbalances, vitamin K<sub>1</sub>, and plasma transfusions should be considered. In humans, liver transplantation has been used successfully in patients with fulminant liver failure. Currently, liver transplantation is not an option for animals poisoned with amanitins.

Diagnosis of amanitin toxicosis is aided by identification of amanitin-containing mushrooms in the environment of the animal. Mushroom pieces found in gastric contents can confirm exposure. Accurate mushroom identification will require consultation with an experienced mycologist. Detection of amanitins in biological specimens is confirmatory, but these tests are not routinely available at diagnostic laboratories. A liquid chromatography–mass spectrometry method was developed and successfully applied to confirm amanitin poisonings in animals and humans (Filigenzi *et al.*, 2007). A competitive enzyme-linked immunosorbent assay was constructed that allows for the detection of  $\beta$ -amanitin in human serum and urine, but this assay is not available in clinical settings (Abuknesha and Maraghkou, 2004). Rapid confirmation of amanitins in suspect exposures assists in the early recognition of exposure, whereas a negative result can prevent unnecessary hospitalization. The well-known reported newspaper test of Wieland or the Meixner test should not be used alone to identify amanitin-containing mushrooms (Beuhler *et al.*, 2004). In suspect cases of amanitin poisoning, serum and urine samples should be collected at various time points beginning as early after exposure as possible. In postmortem presentations, liver and kidney samples are suitable for testing to confirm exposure. The suspect mushroom or vomited gastrointestinal contents should also be saved for further analysis.

Differential diagnoses in dogs and cats with a clinical presentation that involves gastroenteritis and hepatic failure include other toxic ingestions, such as microcystins, cocklebur, cycad palm, aflatoxin, xylitol, ricin, abrin, gyromitrin, and acetaminophen overdose. The history and geographic environment of the animal can help to eliminate most of the toxicant differentials on the list.

## HYDRAZINES

*Gyromitra* species are members of the false morel family, and *Helvellaceae* are usually found under conifers, aspens, and sometimes around melting snowbanks. The species most commonly associated with poisoning and studied in most detail is *G. esculenta*, but the toxins have been found in other species of *Helvellaceae* (Viernstein *et al.*, 1980), including *G. gigas*, *G. fastigiata*, *G. infula*, *Helvella crispa*, and *H. lacunose*. The toxins associated with false morel poisoning are hydrazine analogs. The toxins are heat labile, volatile, and water soluble (Michelot and Toth, 1991). The process of boiling and drying decreases, but does not completely eliminate, the toxin concentrations (Pyysalo, 1976). People who eat only a few of the cooked mushrooms may ingest sufficiently detoxified amounts so as to remain symptom free. This has caused misunderstandings among people with regard to the potential lethality of these mushrooms. Animals generally eat raw mushrooms; therefore, any exposure to these mushrooms may result in serious morbidity and mortality. Poisoning by some species of *Helvella*, *Verpa*, *Morchella*, *Peziza*, *Disciotis*, and *Sarcosphaera* closely resembles the syndrome caused by gyromitrin. It has been speculated that these mushrooms also contain hydrazines, although analysis has yet to confirm the presence of these toxins (Lincoff and Mitchel, 1977b).

### Pharmacokinetics/toxicokinetics

There is very limited information available. Toxicosis can result after oral and inhalation exposure. Ingestion of gyromitrin (acetaldehyde *N*-methyl *N*-formylhydrazine)-containing mushrooms results in the hydrolysis of gyromitrin to *N*-methyl-*N*-formylhydrazine, which is further metabolized to monomethylhydrazine. The degree of hydrolysis is dependent on the pH in the stomach, but it is not complete (Wright *et al.*, 1978). Inhalation of the fumes during the cooking process can also result in poisoning. Once hydrazines reach the liver, they are further metabolized to reactive intermediates, such as methyl cations and free methyl radicals (Gannett *et al.*, 1991).

### Mechanism of action

Gyromitrin is considered a gastrointestinal irritant leading to vomiting, abdominal pain, and diarrhea 6–12 h after ingestion (Coulet and Guillot, 1982). The principal toxin responsible for convulsions seen in severe cases is monomethylhydrazine, which inhibits pyridoxal phosphokinase resulting in decreased pyridoxal 5-phosphate concentrations (Lheureux *et al.*, 2005). Depletion of pyridoxal 5-phosphate leads to decreased  $\gamma$ -aminobutyric acid (GABA) synthesis and an increase in glutamic acid concentrations. Based on a study of mice in which GABA concentrations in the brain were not significantly decreased after methylhydrazine exposure (Maynert and Kaji, 1962), other mechanisms have been proposed. In addition to the gastrointestinal irritation and neurotoxicity, liver and renal failure as well as hemolysis have been described. *N*-methyl-*N*-formylhydrazine inhibits cytochrome P450 and glutathione-metabolizing enzymes (Braun *et al.*, 1979), and it can cause liver necrosis. However, the highly reactive metabolites, such as methyl cations, generated in the liver may significantly contribute to the hepatic injury. Furthermore, the hydrazine analogs present in false morels are carcinogenic in laboratory animals (Toth and Gannett, 1994). Only one case report exists in the veterinary literature. A 10-week-old dog vomited 2 or 3 h after chewing on a mushroom later identified as *G. esculenta* (Bernard, 1979). Six hours post-ingestion, the dog was lethargic, became comatose, and died 30 min later. Histopathological findings included renal tubulonephrosis, periascinar hepatic degeneration, and erythrophagocytosis.

### Toxicity

In humans, there seems to be great individual variability with regard to the toxicity of false morel. In mice, the oral LD<sub>50</sub>s of gyromitrin, *N*-methyl-*N*-formylhydrazine, and monomethylhydrazine are 344, 118, and 33 mg/kg, respectively (Wright *et al.*, 1978). In humans, the estimated lethal dose of gyromitrin is 20–50 mg/kg for adults and 10–30 mg/kg for children (Schmidlin-Meszaros, 1974). Toxicity information for dogs or cats does not exist. Gyromitrin concentrations in fresh *G. esculenta* are estimated to be 0.12–0.16%.

### Treatment

Most humans only develop mild gastrointestinal symptoms and recover fully within several days after exposure. Management is principally supportive. Early decontamination is often not possible because of the delayed onset of clinical signs. Administration of

activated charcoal has been recommended, although efficacy data do not exist. Correction of fluid and electrolytes is an important measure along with the administration of pyridoxine. The recommended dose in humans is 25 mg/kg i.v. over 15–30 min. The dosing can be repeated but should not exceed more than 20 g/day. Although pyridoxine can successfully control seizure activity, it has no benefit in preventing liver injury. In dogs, pyridoxine has been used successfully for non-mushroom toxin-induced seizure activity. It can be used alone or in combination with diazepam, but combination therapy has better efficacy than pyridoxine alone (Villar *et al.*, 1995). The dose for dogs is 75–150 mg/kg body weight, given i.v., during acute phases of seizure activity. Diazepam is given to dogs and cats at 0.5–1.0 mg/kg i.v. to effect. Phenobarbital is not recommended for seizure control because of its cytochrome P450-inducing capability. Administration of folinic acid has been recommended in humans, but controlled studies have not been performed. *N*-acetylcysteine can be considered. The dosing is the same as that given in acetaminophen-induced hepatic injury. Hemodialysis has been reported in the literature, but its role in removing gyromitrin or its toxic metabolites is not known.

Diagnosis of gyromitrin toxicosis is aided by identification of gyromitrin-containing mushrooms in the environment of the animal. Identification by a mycologist is important to distinguish between the true and the false morels. Detection of gyromitrin, hydrazine analogs, or metabolites in mushrooms or biological specimens is not routinely available (Arshadi *et al.*, 2006). Diagnosis is mainly based on clinical and clinicopathological findings and also mushroom identification.

## MUSCARINIC AGENTS

*Inocybe* and *Clitocybe* spp. contain the highest concentrations of muscarine, but lower concentrations are found in many other genera, including *Entoloma* and *Mycena* (Young, 1994). Muscarine was first discovered and characterized in *A. muscaria*, but concentrations of muscarine are only approximately 0.0003% (Eugster and Schleusener, 1969). In comparison, *Inocybe* and *Clitocybe* species have muscarine concentrations between 0.1 and 0.33%. *Inocybe* and *Clitocybe* species have worldwide distributions and are relatively common. *Inocybe* species grow in association with either conifers or broad-leaved trees. *Clitocybe* species grow on forest litter or grassland humus. The risk of poisoning remains after cooking because of the heat stability of muscarine. There is one published report of *Inocybe phaeocomis* poisoning in a dog (Yam *et al.*, 1993) and reported death of a dog presumably from *Inocybe* sp. exposure (Beug, 2009).

## Pharmacokinetics/toxicokinetics

Limited data are available. The naturally occurring form of muscarine is the (L)+ form. In general, quarternary ammonium compounds are poorly absorbed after oral exposure. Once absorbed, muscarine is quickly distributed throughout the body, and clinical signs develop within 30 min to 2 h. Because of its quaternary configuration, muscarine does not cross the blood–brain barrier, and its cholinergic effects are entirely peripheral. A portion of the ingested muscarine is eliminated unchanged in urine, but detailed toxicokinetic studies have not been performed.

## Mechanism of action

Muscarine acts in the peripheral nervous system, where it competes with acetylcholine at its receptor binding sites. The muscarinic cholinergic receptors are found in the heart in both its nodes and its muscle fibers, in smooth muscles, and in glands. They do not occur in skeletal muscles. Once bound to the receptor, muscarine mimics the effect of acetylcholine. Muscarine is not susceptible to inactivation by acetylcholinesterase (Young *et al.*, 1994), and uncontrolled overstimulation of receptors occurs. Clinical signs appear within a few hours and include salivation, lacrimation, vomiting, diarrhea, abdominal pain, miosis, and bradycardia (Lurie *et al.*, 2009). Clinical signs in the dog eating *I. phaeocomis* were observed 3 h after exposure and included salivation, diarrhea, vomiting, depression, and collapse (Yam *et al.*, 1993).

## Toxicity

The i.v. LD<sub>50</sub> of muscarine in mice is 0.23 mg/kg (Waser, 1961). The lethal dose of muscarine in humans is estimated to be between 180 and 300 mg. Ingestion of a single mushroom containing 0.33% muscarine on a dry weight basis can be lethal (Bresinsky and Besl, 1990a).

## Treatment

Treatment includes early decontamination, administration of activated charcoal, and fluid rehydration. If life-threatening clinical signs are present, atropine should be administered. After giving a test dose to determine its efficacy, atropine can be given repeatedly until symptoms are abolished or until cessation of salivation. Doses in dogs and cats are 0.2–2.0 mg/kg, where one-fourth of the dose is given i.v. and the remainder is given either subcutaneously or intramuscularly. Other criteria for therapeutic endpoint with atropine include ease of



respiration and lack of respiratory secretions. Mydriasis is not an indicator of its effectiveness. Because atropine also competes with acetylcholine at the receptors, ongoing treatment must be carefully monitored for its anticholinergic effects, including tachycardia, gastrointestinal stasis, severe behavioral changes (e.g., delirium), and hyperthermia. The dose of atropine should be reduced or discontinued with these adverse effects.

## ISOXAZOLES

Poisoning in this group is attributed to the heat-stable isoxazoles derivatives, ibotenic acid and muscimol. *Amanita pantherina* (panther cap or panther agaric) and *A. muscaria* (fly agaric) are most commonly associated with poisonings in humans in this group (Hall and Hall, 1994). The mushrooms grow from summer to autumn in coniferous and deciduous forests, and they are abundant in the Pacific Northwest, where they are often found under Douglas fir trees. Other species containing these toxins include *A. gemmata*, *A. smithiana*, *A. strobiliformis*, and *Tricholoma muscarium* (Lincoff and Mitchel, 1977c). There are a few reports of poisoning in dogs (Naude and Berry, 1997; Beug, 2009) and cats (Ridgway, 1978) after ingesting *A. pantherina*. Ingestion of *A. muscaria* presumably resulted in clinical signs in a horse (Beug, 2009).

### Pharmacokinetics/toxicokinetics

Definitive data on the toxicokinetics of muscimol and ibotenic acid have not been established. Based on the rapid onset of clinical signs after oral exposure, rapid absorption of toxins is suspected. Once absorbed, muscimol and ibotenic acid appear to cross the blood-brain barrier by an active transport system. Ibotenic acid decarboxylates to form muscimol in the stomach, liver, and brain (Nielsen *et al.*, 1985). Muscimol and ibotenic acid can be detected in urine within 1 h of exposure (Ott *et al.*, 1975; Merova *et al.*, 2008).

### Mechanism of action

The major toxins are muscimol and ibotenic acid, but other active substances have been identified, although with minor pharmacological activities. Ibotenic acid is known to act on glutamic acid receptors in the central nervous system (CNS) and produces an excitatory action (Cleland, 1996), whereas muscimol acts on GABA<sub>A</sub> receptors and has a depressant action (Chebib and Johnston, 1999). Although both muscimol and

ibotenic acid are present in mushrooms, muscimol is further derived from ibotenic acid by spontaneous decarboxylation that can occur during dehydration of the mushroom, during digestion in the stomach, or after absorption in a variety of tissues. Therefore, muscimol is likely the major toxin resulting in clinical signs. On activation by muscimol, the membrane permeability for anions increases, usually resulting in a slight, short-lasting hyperpolarization and associated decreased excitability of the receptive neuron. Effects on the CNS are similar to those produced by therapeutic doses of diazepam.

Clinical signs of muscimol toxicosis begin within 30 min to 2 h after ingestion and have been termed the “pantherine-muscaria” syndrome in humans, which is characterized by mydriasis, dryness of mouth, ataxia, confusion, euphoria, dizziness, and tiredness. Gastrointestinal signs are not consistently seen in cases of isoxazole poisoning. Full recovery is expected within 1 or 2 days. In cats, clinical signs have been observed within 15–30 min after ingestion of *A. pantherina* (Ridgway, 1978). After a brief period of sedation, cats experienced a 4-h-long state of excitement with pronounced muscle spasms, followed by a deep sleep. Cats are expected to fully recover within 24 h after ingestion, especially if decontamination measures are taken. In dogs, clinical signs observed after ingestion of *A. pantherina* include disorientation, opisthotonus, paresis, seizures, paddling, chewing movements, miosis, vestibular signs, respiratory depression, coma, and death (Hunt and Funk, 1977; Naude and Berry, 1997). Recoveries are recorded within 12–24 h after aggressive supportive care measures, including mechanical ventilation during periods of respiratory depression; however, death was reported in several dogs. Similar clinical signs were reported in a dog that survived *A. muscaria* poisoning (Martin, 1956).

### Toxicity

In mice, the oral LD<sub>50</sub> is 22 mg/kg for muscimol and 38 mg/kg for ibotenic acid (Hall and Hall, 1994). In rats, the oral LD<sub>50</sub> is 45 mg/kg for muscimol and 129 mg/kg for ibotenic acid. The intraperitoneal LD<sub>50</sub> of muscimol is 2.5 mg/kg in mice and 3.5 mg/kg in rats. In humans, the toxic threshold is estimated to be 6 mg of muscimol and 30–60 mg of ibotenic acid (Halpern, 2004). The concentration of ibotenic acid in *A. muscaria* was estimated at 100 mg/kg fresh weight, whereas the concentration of muscimol was less than 3 mg/kg fresh weight. Thus, an average-sized fruit body of *A. muscaria* weighing 60–70 g can contain a toxic concentration. Toxicity data for dogs are not available. However, postmortem examination of puppies indicated that the ingestion of a single *A. pantherina* can be lethal (Hunt and Funk, 1977).

## Treatment

Treatment of exposed animals is mainly symptomatic and supportive. Decontamination measures should be considered in recent exposures, although emetics are only recommended in animals that are not at risk for developing aspiration pneumonia. Specific measures to control seizures are not without complication. Benzodiazepines, as GABA agonists, may potentiate any CNS depression. Therefore, when diazepam is used, CNS and respiratory depression may be severe and prolonged, necessitating the use of mechanical ventilation. As long as ventilation is maintained adequately, the prognosis for recovery is good. Diazepam can be given to dogs and cats at 0.5mg/kg i.v. to effect and can be repeated as needed every 10min for up to three doses. Other drugs used to control seizures include phenobarbital and pentobarbital. Phenobarbital is dosed at 6mg/kg i.v. Pentobarbital is given at 5–15mg/kg i.v. to effect to dogs and cats. These, too, are agonists at the GABA receptors, and they can potentiate CNS and respiratory depression. Careful monitoring of the animal's oxygenation status is vital until the animal is fully awake and alert. Supplemental oxygen can be used if necessary. General supportive measures of unconscious animals include maintaining hydration with i.v. fluids, maintaining the airway free of respiratory secretions, and frequent position changes to prevent decubitus skin ulcerations. In humans, the use of atropine is contraindicated because of the atropine-like clinical presentation in poisonings.

## PSILOPIN AND PSILOCYBIN

Mushrooms that contain psilocybin are commonly referred to as hallucinogenic or magic mushrooms. *Psilocybe*, *Panaeolus*, *Conocybe*, and *Gymnopilus* are the four genera in North America that contain psilocybin (Smolinske, 1994). Many of these mushrooms are coprophilic and grow in fields and animal pastures, particularly in the northwestern and southeastern United States. The majority of mushrooms contain only psilocybin, but some, such as *Psilocybe cyanescens*, contain both psilocybin and psilocin. The concentrations of psilocybin and psilocin are influenced by growth conditions, geographic location, storage conditions, and species. Species commonly found in the Pacific Northwest contain between 1.2 and 16.8mg/kg psilocybin on a dry weight basis. If psilocin is present, concentrations may reach up to 9.6mg/kg on a dry weight basis (Smolinske, 1994). Psilocin and psilocybin are sensitive to heat. Some mushrooms in this group also

contain other pharmacologically active substances, such as serotonin and tryptophan. There is only one published report of hallucinogenic mushroom ingestion in a dog (Kirwan, 1990).

## Pharmacokinetics/toxicokinetics

Psilocybin is a prodrug and is rapidly dephosphorylated to psilocin. Dephosphorylation can take place in a variety of tissues, but high activity has been identified in kidney and liver of rodents (Horita and Weber, 1961) and in plasma of humans (Grieshaber *et al.*, 2001). However, the general assumption is that complete conversion of psilocybin to psilocin occurs prior to absorption into the systemic circulation (Laatsch, 1996). In humans, the absolute bioavailability of psilocin liberated from orally administered psilocybin was estimated to be  $52.7 \pm 20\%$  (Hasler *et al.*, 1997). Once absorbed, psilocin is further metabolized in plasma to 4-hydroxytryptophole (4HT) and 4-hydroxyindole-3-acetic acid (4HIAA). Psilocin crosses the blood-brain barrier and concentrates in brain tissue (Horita and Weber, 1961). Psilocin is excreted unchanged or as psilocin-glucuronide in urine and to some extent unchanged via the bile (Hasler *et al.*, 2002).

## Mechanism of action

Psilocin is the pharmacologically active metabolite of psilocybin. Because of its structural similarity to serotonin, psilocin stimulates serotonin receptors in the CNS (McKenna *et al.*, 1990). Psilocin has affinity for 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>2C</sub> receptors (Halberstadt *et al.*, 2010). Activation of 5-HT<sub>2A</sub> receptors leads to increased cortical activity via glutamatergic excitatory postsynaptic potentials (Aghajanian and Marek, 1997). Activation of 5-HT<sub>1A</sub> receptors results in the inhibition of pyramidal cell activity (Puig *et al.*, 2005). In addition, psilocin may have peripheral effects that involve serotonergic receptors. In humans, psilocin's psychoactive effects are similar to those produced by LSD, are observed within 20–30 min of ingestion, and include visual hallucinations, intensified hearing, and incoordination. Other autonomic-mediated effects include increased heart rate, increased blood pressure, mydriasis, tremors, and increased temperature. The effects can last up to 8 h, but hallucinogenic activity rarely exceeds 1 h. Clinical signs in dogs include ataxia, vocalization, overt aggression, nystagmus, and increased body temperature (Kirwan, 1990). In contrast to the CNS effects after exposure to isoxazoles, there is no subsequent coma. Sedation may be necessary until behavioral signs resolve.

## Toxicity

In many countries (e.g., the United States, Great Britain, and Germany), psilocybin and psilocin are classified as controlled substances. In humans, oral exposure to 10–20 mg of psilocybin can cause mood changes and hallucinations. Information regarding lethal doses in animals is not found in the literature.

## Treatment

The management of hallucinogenic mushroom poisonings is primarily supportive, and in most cases treatment is not necessary. Gastric emptying procedures have not proven beneficial and are not recommended. The effect of activated charcoal in poisonings is not known, but activated charcoal administration can be considered. If severe neurologic signs such as seizures occur, diazepam is considered the first-line medication. Diazepam can be given to effect to dogs and cats at 0.5–1.0 mg/kg i.v. in increments of 5–10 mg. If diazepam is unsuccessful, subsequent seizures can be controlled with phenobarbital at 6 mg/kg to effect. In addition, control of body temperature is an important factor in symptomatic care.

Diagnosis of psilocybin exposure is confirmed by the detection of psilocin and psilocin-glucuronide in urine, serum, and blood. A number of methodologies are available and are routinely used in forensic investigations (Kamata *et al.*, 2003; Albers *et al.*, 2004; Laussmann and Meier-Giebing, 2010). Because of the infrequent presentation of hallucinogenic mushroom poisoning in animals, these methodologies are not routinely available at veterinary diagnostic laboratories.

## GASTROINTESTINAL IRRITANTS

This group includes mushrooms that result in gastroenteritis as the primary clinical sign. There are very few reports in the veterinary literature. Genera included are *Agaricus*, *Boletus*, *Chlorophyllum*, *Entoloma*, *Lactarius*, *Omphalotus*, *Rhodophyllum*, *Scleroderma*, and *Tricholoma*. The specific toxins in most have not been identified (Spoerke, 1994).

One of the mushrooms in this group commonly reported to cause poisoning is *Chlorophyllum molybdites*. This mushroom is commonly found in the United States except in the colder, northern areas, and the principal toxin has not been clearly identified. Clinical signs of nausea, vomiting, and diarrhea appear 1–6 h after ingestion in humans, and there is complete recovery within 24–48 h (Blayney *et al.*, 1980). A dog with *C. molybdites* poisoning had clinical signs of drooling and diarrhea,

whereas a horse that had presumably eaten several bites of *C. molybdites* died (Beug, 2009). *Omphalotus olearius*, *Omphalotus subilludens*, and *Lampteromyces japonicus* contain illudin S (Bresinsky and Besl, 1990b).

Illudin S is a sesquiterpene with a unique chemical structure. In humans, vomiting and diarrhea occur 1–2.5 h after ingestion (French and Garrettson, 1988). In a pot-bellied pig, death was reported 5 h after it ingested a fruiting body of *Scleroderma citrinum* (Galey *et al.*, 1990). The pig vomited and collapsed 20 min after exposure and remained weak and recumbent until death. The toxins in *S. citrinum* have not been characterized. In *Boletus satanas*, lectins may contribute to serious gastroenteritis in humans. Lectins, which are storage proteins that may play a role in plant defense, are widely distributed in many species of mushrooms (Wang *et al.*, 1998).

Poisonings by mushrooms of this group are rarely fatal; hence, they are likely to be underreported by owners and rarely recorded in the literature. After a usually short latent period of 15 min to 2 h after ingestion, an animal may present with vomiting, diarrhea, and abdominal pain. Usually, these clinical signs resolve spontaneously within a few hours, but they may last 1 or 2 days. The clinical signs may resemble any other common cause of gastroenteritis in small or large animals, including bacterial and viral infections, sudden diet changes or eating spoiled foods, and inflammatory mediated syndromes such as acute pancreatitis. The diagnostic challenge is that many of the more toxic mushrooms cause initial gastrointestinal signs. Cyclopeptides cause vomiting and diarrhea after a lag period similar to that seen with the gastrointestinal irritants. Therefore, any animal presenting with gastrointestinal signs after a known history of mushroom ingestion must be carefully assessed.

Treatment is entirely nonspecific and supportive. Vomiting is a hallmark of poisoning by gastrointestinal irritant mushrooms. Thus, in most cases, the stomach has already been emptied, and emetics are not necessary. Activated charcoal is thought to adsorb most of the toxins in this group and should be administered orally unless there is protracted vomiting. There are no specific antidotes for the toxins in this group. Treatment is aimed at rehydration and correction of serum electrolyte abnormalities.

## RENAL TOXIC MUSHROOMS

Several cyclopeptide-containing mushrooms cause renal failure without any hepatic insult. Mushrooms of the genus *Cortinarius* contain the cyclopeptide orellanine (Frank *et al.*, 2009). In North America, there are only a

few reported cases in humans, but poisonings occur with much greater frequency in western and central Europe. In humans, symptoms may not appear for 2–20 days after ingestion. In general, gastrointestinal signs occur a few days after exposure, and renal failure develops 4–15 days after ingestion. Hemodialysis may be necessary until renal function gradually improves. There are no reports of orellanine poisoning in animals.

*Amanita smithiana* has resulted in delayed renal failure in humans without evidence of hepatic dysfunction (West *et al.*, 2009). The toxin is allenic norleucine. Key clinical features are vomiting and abdominal pain with little or no diarrhea within a few hours of ingestion. Renal failure develops 1–4 days after exposure and may require several weeks of hemodialysis before recovery. There are no reports of *A. smithiana* poisoning in animals, but it is a common mushroom in the Pacific Northwest.

## RAMARIA FLAVO-BRUNNESCENS

This mushroom is found in North America, Australia, China, Brazil, and Uruguay and has caused poisoning in cattle and sheep (Kommers and Santos, 1995). *Ramaria flavo-brunnescens* is exclusively found in eucalyptus woods, and thus the poisoning has been termed “eucalyptus sickness.” Clinical signs have been observed as early as 3 days after exposure but may be delayed until 6 days post-exposure. Typically, animals develop anorexia, salivation, diarrhea, and recumbency. This is followed by alteration in keratinization, which becomes apparent by loss of hair and hooves, similar to what is seen in chronic selenosis. The toxins have not been identified but are most likely volatile and interfere with the incorporation of sulfur-containing amino acids.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Overall, the number of reported mushroom poisonings in animals is low, although this is likely a result of the lack of methods to confirm exposure. In humans, most cases are diagnosed by positive identification of the suspect mushroom, which is often impossible in veterinary medicine. The chances of obtaining an intact and representative mushroom are slim because animals are often left unattended and a history of ingestion is not available. The development of new analytical techniques to identify mushroom toxins in biological samples of poisoned animals will provide insight into the true frequency of mushroom poisonings. Currently, therapeutic

measures are primarily based on both mechanisms of toxicity and clinical signs. Rapid toxin identification would allow for a confirmed, early diagnosis, which is especially important in cases in which intensive care is indicated. To develop analytical techniques for specific mushroom toxins, further research is necessary in the area of toxin characterization and toxicokinetics. Although thousands of mushrooms exist worldwide, only a few have been researched in-depth. It is also important to improve our knowledge of the efficacy of commonly recommended, but poorly evaluated, therapeutic procedures.

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## *Datura* species and related plants

Vinny Naidoo

### INTRODUCTION

The family Solanaceae contains several genera that are toxic due to the presence of the alkaloids hyoscyamine, scopolamine (hyoscine), nicotine, and solanine. Of these, hyoscyamine and scopolamine, the toxic tropane alkaloids, are found within the genera *Atropa* (belladonna), *Hyoscyamus* (henbane), *Brugmansia* (angel's trumpet), and *Datura* (jimsonweed). Although all of these genera are of toxicological importance, only the genus *Datura* appears to be of veterinary significance because the others are uncommonly associated with accidental poisoning. Within the *Datura* genera, the species of concern are *D. stramonium*, *D. ferox*, *D. inoxia*, *D. quercifolia*, *D. wrightii*, and *D. metel* (Knight and Walter, 2003; Molyneux and Panter, 2009), with the first two being of most significance. *Datura stramonium* L. (jimsonweed, common thorn apple, or Jamestown weed) (Figure 85.1) and *D. ferox* L. (large thorn apple) (Figure 85.2) both enjoy a cosmopolitan distribution worldwide and are annual pioneer weeds on wastelands. They are particularly troublesome, serious invaders on fertilized soil with annual crops (Anderson and Henderson, 1966; Williams, 1980).

The *Datura* spp. are very similar morphologically and are characterized by their malodorous leaves (leading to one of their common names of stink leaf) and spiny fruit capsules that are erect and ovoid. Although the two main species *D. ferox* and *D. stramonium* look very similar, they can be differentiated by the fruit capsule because *D. ferox* has the longer spines between the two. The flowers of the plants are erect, funnel shaped, and white for the *D. ferox* and white, mauve, or purplish for *D. stramonium*. The stems are green, except a purple-stemmed variety



**FIGURE 85.1** *Datura stramonium* L. (jimsonweed, common thorn apple). (Courtesy of the South African National Biodiversity Research Institute (SANBRI), Pretoria, South Africa.)

can occur with the latter. The seeds of the two species are also identical in shape and are flattened black kidney shaped with characteristic pitted exterior (Anderson and Henderson, 1966). Although the seeds





FIGURE 85.2 *Datura ferox* L. (large thorn apple). (Courtesy of SANBRI, Pretoria, South Africa.)

are identical morphologically, the seeds of *D. ferox* are larger at 14.1 versus 6.6 mg. A third species, *D. innoxia* Mill. subspecies *innoxia* (downy thorn apple or moon-flower; Figure 85.3), is primarily an invader of waste areas and sandy riverbanks, and occasionally it can also be an invader of crops. Although it also produces large tubular white flowers, in contrast to the two previous species, the flower is softly gray velvety on all parts. In addition, the fruit capsule is densely covered in small spines, with the seeds being brown and of a similar shape and size as those of *D. ferox*.

*Datura stramonium* can be further divided into four varieties: *D. stramonium* var. *stramonium*, *D. stramonium* var. *tatula*, *D. stramonium* var. *inermis*, and *D. stramonium* var. *godroni*. There is minimal morphological difference between the four species; for example, *D. stramonium* var. *stramonium* has white flowers, whereas *D. stramonium* var. *inermis* has purple flowers, the latter being a dominant purple gene effect, and *D. stramonium* var. *inermis* has a smooth capsule due to a recessive gene for the characteristic (Berkov *et al.*, 2006). However, the four strains do differ in their toxin content.

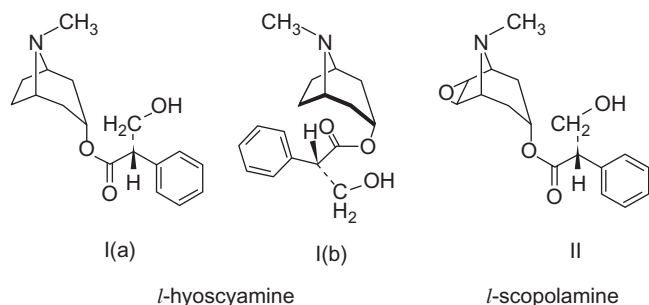


FIGURE 85.3 *Datura* subspecies *innoxia* Mill. subspecies *innoxia* (*D. meteloides* Dunal) (downy thorn apple). (Courtesy SANBRI, Pretoria, South Africa.)

## CHEMISTRY

The toxins that occur within the plant are known as the tropane alkaloids. These alkaloids basically consist of a bicyclic seven-member tropane alkaloid ring esterified through its hydroxyl group at C-3 with the carboxyl group of L-tropic acid (Figure 85.4). The toxic L-hyoscyamine predominates over scopolamine in *D. stramonium*, whereas *D. ferox* contains predominantly scopolamine (Ketchum and Sidell, 1997). L-Hyoscyamine racemizes in extraction to form a mixture of DL-hyoscyamine (known as atropine), which is only 50% active because the D-isomer is devoid of activity (Molyneux and Panter, 2009). Chemically, the toxins are produced in the roots and are translocated via the xylem to the aerial parts, where they are stored in vacuoles (Wink, 2009).





**FIGURE 85.4** (I) Structures of hyoscyamine (conventional (a) and stereo-specific (b)) and (II) scopolamine (conventional).

**TABLE 85.1** Concentration and percentage recovery of atropine and scopolamine from wheat flour before and after baking in bread

Material	Atropine		Scopolamine	
	mg/g mixed flour	% recovery	mg/g mixed flour	% recovery
Wheat flour + 12% jimsonweed seed	0.406	100	0.115	100
Bread crumbs	0.304	75	0.1	87
Bread crust	0.335	82	0.083	72

From Friedman and Levin (1999).

The scopolamine and atropine toxins within the plant are very stable and are not destroyed by normal processing methods. List and Spencer (1979) demonstrated that processing contaminated soybean meal cake resulted in approximately 90% of the available atropine and scopolamine remaining in the soybean cake. The remaining toxins were found in the oil. However, the atropine and scopolamine in the oil were reduced by 93% through the caustic refining process. The authors concluded that this was an expected finding because the tropane alkaloids were known to hydrolyze at alkaloid pH (List and Spencer, 1979). It was also demonstrated by Friedman and Levin (1999) that baking contaminated flour did not reduce the toxin levels to any substantial concentration (Table 85.1).

## TOXICITY AS RELATED TO THE PLANT PART

Although the entire plant is toxic (Table 85.2), the toxicity is most commonly associated with the ingestion of

**TABLE 85.2** Alkaloid content ( $\mu\text{g}/\text{mg}$ ) in *Datura stramonium*

Samples	Young plant		Adult plant	
	Atropine	Scopolamine	Atropine	Scopolamine
Small leaves	0.156	0.073	0.165	0.016
Medium leaves	0.831	0.047	0.15	0.022
Large leaves	0.228	0.035	0.134	0.044
Stems	0.915	0.129	0.001	—
Roots	0.121	0.014	—	—
Flowers	0.229	0.106	0.27	0.066
Pericarp	0.001	—	0.001	—
Seeds	0.17	0.012	0.387	0.089

From Miraldi *et al.* (2001).

Flowers: represent buds for young plants and open flower for adults; fruits: immature fruits for young plants and mature fruits for adults.

seeds as a contaminant of commercial grain crops. This is due to their rapid growth when fields are invaded, which results in concurrent harvesting of the seed with the corn. The real problem with toxicity lies with the seeds, which have the same specific gravity as the corn and are thus not easily separated unless shifted in different layers. The latter is the reason why many countries have legislation controlling the number of seeds allowable in a particular crop (corn, sorghum, soybeans, and linseed). The plant itself is very rarely directly toxic to animals because it is not consumed due to poor palatability (Ralphs, 2002). Toxicity usually only results when animals are forced to eat the plant when exposed to poor grazing on pastures or, alternatively, when the dried plant parts contaminate the hay of intensively fed animals.

Nonetheless, despite toxicity being possible irrespective of the plant part consumed, the content of scopolamine (hyoscyne) and hyoscyamine does differ per plant part (Rhodehamel and Stuart, 1921; Miraldi *et al.*, 2001). The highest concentrations are found in the leaves, flowers, and seeds of the mature plant (Table 85.2). The reason for this distribution most likely rests in a need for these structures to be protected from consumption by both herbivorous mammals and insects. Being an annual plant, it does not store resources that could be used to reestablish biomass after consumption, with the result that the plant has developed a secondary mechanism to decrease foliage consumption (Valverde *et al.*, 2003).

In addition, the toxin content is related to the *Datura* plant species with *D. stramonium* containing hyoscyamine and scopolamine at approximately equal ratios, whereas *D. ferox* contains predominantly scopolamine at a ratio of 98:2.

## TOXICODYNAMICS AND TOXICOKINETICS OF TROPANE ALKALOIDS FROM *DATURA* SPP.

Both atropine and scopolamine function by being non-selective antagonists of the muscarinic receptors in the body, thus preventing acetylcholine esterase from having an effect. The muscarinic receptors affected are present on neuroeffector junctions supplying the smooth and cardiac muscle, glandular tissue, certain peripheral ganglia, and the central nervous system (Brown and Taylor, 2001); that is, the clinical effects are always multisystemic due to the distribution of the specific receptor.

The pharmacokinetics of atropine and scopolamine have not been elucidated from the plant itself (i.e., the rate of release). The pharmacokinetics of the individual toxins, such as atropine and scopolamine, have been characterized in people. In an anesthetic study by Saarnivaara *et al.* (1985), children were treated with atropine at 0.03 mg/kg to determine the onset of the effect of the orally administered atropine. The atropine was characterized by a  $C_{\max}$  of 6.7 nmol/L, with a corresponding  $T_{\max}$  of 2 h. The clinical onset of satisfactory antispasmodic effect occurred within 70 min of administration (Saarnivaara *et al.*, 1985). Elimination half-life is reported to be 4 h (Royal Pharmaceutical Society of Great Britain, 2010).

The pharmacokinetics and bioavailability of scopolamine were evaluated in six healthy male subjects receiving 0.4 mg of the drug by either oral or intravenous administration. Scopolamine was characterized by a  $C_{\max}$  of  $528.6 \pm 109.4$  pg/mL, and the half-life of elimination was  $4.5 \pm 1.7$  h. The oral absolute bioavailability, however, was low at 10.7–48.2% (Kanto and Klotz, 1988).

Although both scopolamine and atropine are equally effective in inducing clinical signs associated with the peripheral nervous system (tachycardia, colic, drying up of secretion, and urinary retention), only scopolamine is responsible for the neurological symptoms of the plant. The reason for this lies in the toxicokinetics of the two molecules because atropine, the more water soluble of the two molecules, fails to reach adequate concentrations within the brain, whereas the more lipid-soluble scopolamine easily crosses the blood–brain barrier to achieve clinically effective concentrations within the central nervous system.

## CLINICAL SIGNS OF POISONING

Although the susceptibility of people to toxicity is unquestionable, there seems to be debate as to which

domestic species is the most susceptible. Piva and Piva (2006) stated that pigs were the most susceptible species, followed by cattle, horses, and chickens. However, based on evaluation of the literature, it appears that the order of susceptibility may realistically be horses > pigs > cattle > poultry based on susceptibility and chance of exposure. Sheep and rabbits are not normally susceptible due to the presence of an esterase enzyme system that rapidly breaks down the alkaloids (Piva and Piva, 2006).

### Equines

In general, toxicity from the ingestion of the growing plant in the grazing animal is not a problem because the plant is often avoided due to its offensive smell. *Datura* poisoning in horses is therefore usually accidental and results from the ingestion of the seed as a contaminant in the grain or the plant as a contaminant of the hay. The latter tends to result from both the inability of the animal to select its food and the loss of the offensive smell as the plant dries.

Toxicity in horses results soon after ingestion. In field outbreaks, clinical signs are reported to occur within 6–24 h of exposure to seeds in the feed (Salyi and Abonyi, 1994). In a more controlled study by Galey *et al.* (1996), four adult mares exposed to air-dried *D. innoxia*, containing 550 mg scopolamine and 740 mg atropine at levels of 0.0125, 0.025, 0.1, and 0.5 g of plant material per kilogram, developed clinical signs consistent with toxicity (gastrointestinal atony, tachycardia, sweating, and colic) 2 h after dosing. Clinical signs failed to resolve by 72 h post-dosing. The alkaloid concentrations peaked within 1 or 2 h after dosing, and the half-life of elimination was determined to be 1.7 and 2.3 h for scopolamine and atropine, respectively, for the lowest dose. An interesting finding was the lack of a relationship between the duration of the clinical signs in relation to the half-life (72 vs. 2.3 h). Although this result was never explained, two plausible explanations are that the effect on the muscarinic receptor system is more prolonged, akin to irreversible antagonism, or, more likely, due to constant low-level absorption from the gastrointestinal tract (i.e., atropine and scopolamine are slowly released as the plant is digested).

A number of field outbreaks from the ingestion of contaminated grain have been reported (Barney and Wilson, 1963; Williams and Scott, 1984; Salyi and Abonyi, 1994; Schulman and Bolton, 1998), all with varied clinical signs. This suggests that toxicity is related to the amount ingested, the toxin concentration within the grain, and, to a degree, individual variation. In the first case, 15 of 34 animals were affected following exposure to cracked maize containing a large number of seeds (Barney and Wilson, 1963). The clinical signs recorded were anorexia,

hyperexcitability, staggers, muscular spasms, frequent urination, mydriasis, and impaired vision progressing to convulsions, seizures, rigor, coma, and death in 11 animals. In the second case, 2 horses fed a meal containing 0.5% *D. stramonium* seeds showed signs of depression, mydriasis, anorexia, tachycardia, polydypsia, polyuria, fever, and fetid diarrhea (Williams and Scott, 1984). In the third report, in which toxicity was assumed to have resulted from exposure to a combination of *D. stramonium* and *D. ferox* in 2 horses, is the first incidence of severe gastrointestinal signs being recorded as 1 horse died from acute gastric dilation and rupture and the other from an unresponsive paralytic ileus (Schulman and Bolton, 1998).

Toxicity via contamination of hay has also been reported (Naude *et al.*, 2005; Gerber *et al.*, 2006). In both cases, toxicity was confirmed by the presence of the toxic plant in the hay. In the first case, *D. stramonium* and *D. ferox* toxicity was suspected from the physical examination of the plant and high-performance liquid chromatography analysis, which demonstrated the presence of scopolamine and hyoscyamine (1600:1500 µg/g) in one of the dried plants and only scopolamine in the other (1100 µg/g). The second case confirmed toxicity by the presence of the plant in the hay and also by the presence of hyoscyamine and scopolamine at 18.5 and 1553 µg/g, respectively, in the urine (Gerber *et al.*, 2006). Clinical signs for both these cases were consistent with that for the seed exposure and included impaction, colic, and paralytic ileus.

The clinical pathological changes induced by *Datura* were evaluated by Binev *et al.* (2006a–c) following the exposure of horses to fresh maize with a high content of jimsonweed. The erythron revealed an erythrocytosis and increased hematocrit in the absence of changes in mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, and mean corpuscular volume, which suggested the induced changes were due to dehydration. The leukon was more revealing and showed an increase in the total leukocytes, metamyelocytes, and band and segmented neutrophil counts, whereas the lymphocyte count decreased. The specific change in the leukon was believed to have resulted from either direct irritation of the mucosa by the toxin or, more likely, the colic that developed. Binev *et al.* also suggested that a failure of the leukon to return to normal within 4 or 5 days of exposure may be a good indicator of an overall poorer prognosis.

The pathology that results from the ingestion of *D. stramonium* seeds appears to be nonspecific. Salyi and Abonyi (1994) reported clinical signs of restlessness, colic, dyspnea, tachycardia, peripheral circulatory insufficiency, constipation, tympany, ileus, dilated pupils, and drying of oral and nasal mucosa. On postmortem, seeds were always present in the stomach in combination with

other nonspecific changes, such as circulatory disturbances, pulmonary emphysema, cardiac dilation, constipation, and, occasionally, intestinal displacement. The nonspecific pathology was related more to the colic that developed rather than specific changes induced by the toxins; that is, congestion and pulmonary pathology are common complications of colic due to increased pressure on the diaphragm by parts of the distended gastrointestinal tract.

## Cattle

Few studies on the clinical signs of *Datura* intoxication are available for cattle. This probably results from the low levels of toxicity seen because animals do not readily consume the plant due to poor palatability; that is, poisonings only result when animals are forced to consume the plants in times of starvation. According to Naude (2007), fresh *Datura* posed little problem in cattle as animals were observed consuming the plants on well-fertilized pastures. Analysis of *D. ferox* revealed 149 and 988 µg/g of hyoscyamine and scopolamine, respectively, whereas *D. stramonium* contained 260 µg/g each of scopolamine and hyoscyamine, which are at the toxic levels. Therefore, this suggests that the rumen is capable of deactivating the ingested tropane alkaloids when given time to habituate.

The clinical signs of hyoscyamine and scopolamine exposure, however, were elucidated in a controlled study in cattle. Nelson *et al.* (1982) exposed heifers to a diet containing 0, 8.8, 880, and 4410 of *D. stramonium* seeds/kg of feed for 14 days. The seeds were shown to contain 0.26% atropine and 0.55% scopolamine, which worked out to a total exposure of 2.49 g atropine and 0.5 g scopolamine/kg/day for the high-dose group. The main clinical sign of anorexia was evident in only the high-dose group and resolved completely within 2 or 3 days. It was speculated that the toxins induced rumen atony that subsequently presented as anorexia. With the induced anorexia, the animal stopped consuming the toxic material and as a result was allowed sufficient time for toxin washout and return to normal health. Other clinical signs seen in the high-dose group were spells of bloat, dry muzzle, miosis, and tenesmus (Nelson *et al.*, 1982).

## Sheep and goats

According to Naude (2007), sheep are very resilient to the effects of *D. stramonium*. In an unpublished study, sheep were dosed by fistula with 50 g/kg of the seeds with a known alkaloid concentration of 0.2% without the development of any clinical signs. The same study also

demonstrated that purified atropine was more effective when given intravenously than orally. The oral dose of 50 mg/kg induced only a marginal increase in papillary dilatation, whereas the intravenous dose of 33 mg/kg induced a marked papillary dilatation and tachycardia. This again supports the information available for cattle that suggests that the rumen does offer protection against orally ingested toxins. Other literature indicates that the protection appears to be from the aromatase enzyme system (Piva and Piva, 2006).

The finding of Naude (2007) does not appear to be a universal finding for this species because a controlled study by El Dirdiri *et al.* (1981) showed a different result. In this study, male desert sheep and Nubian goats were dosed daily with fresh leaves or fruit until death resulted. The sheep ( $n = 2$ ) were dosed with fruit or leaves at 10 g/kg for the fruit and 1 g/kg for the leaves for an intended 61 days. Clinical signs were evident 2–6 days after dosing and continued until death at 12–38 days for fruit, whereas signs were slower to develop in the leaf group, with initiation at 35 and 45 days and continuing until death of the first animal on day 45 or until scheduled death for the second. This trend was evident in the goats ( $n = 4$ ), which were similarly dosed with leaves or fruit at 10 g/kg/day or fruit at 2.5 g/kg/day for a longer period of 136 days. Clinical signs were evident 2–101 days after dosing and continued until death at 30–136 days for the leaf group, whereas signs were slower to develop for the seed group, with initiation at 36–110 days and continuing until the death of the first animal on day 30 or until scheduled death for the remaining animals. The signs noted in both species were reduced water intake, anorexia, intermittent hyperesthesia, tremors, drowsiness, and recumbence. Histological evaluation revealed focal necrosis and fatty vacuolation of centrilobular hepatocytes and renal tubular degeneration.

## Springbok

Lideque and Scheepers (1992) reported that a group of Springbok (*Antidorcas marsupialis*) in the northern Namibian desert area were forced to graze almost exclusively on dense stands of the alien invader *D. inoxia*. Two adult male animals from this group exhibited abnormal behavior, having an unsteady gait and a delayed response to visual and auditory stimuli allowing the observer's vehicle to approach to a quarter of the distance at which these antelope usually fled.

## Pigs

As hindgut fermenters, it is not surprising that pigs are susceptible to tropane alkaloid toxicity because the toxin is preferentially absorbed from the upper gastrointestinal

tract. It is also not surprising that pigs are commonly involved in toxicity because their diets are intensive and consist typically of maize in the ration. Clinical signs in pigs are also muscarinic associated. Reported signs include ataxia, lethargy, docility, drowsiness, papillary dilatation, and possible even gas distention of the gastrointestinal tract (Keeler, 1975).

In the 1970s, it was speculated that *D. stramonium* could be teratogenic and induce arthrogryposis in piglets after the abnormal occurrence of this clinical sign in piglets born to sows exposed to *D. stramonium* (the sows did demonstrate classical signs of *Datura* exposure) (Leipold *et al.*, 1973). The deduction was later shown to be incorrect by Keeler (1984), who dosed pregnant sows with dried *D. stramonium*. For this study, animals were dosed for 40 ( $n = 5$ ) and 10 ( $n = 5$ ) days at 1.2–1.7 g/kg/day, which approximates to 1.8–2.6 mg/kg of tropane alkaloid. The study recorded one fatality at the highest dose, which indicates that the exposure concentration was sufficiently high (Keeler, 1984).

Worthington *et al.* (1981) fed two groups ( $n = 4$ ) with ground *Datura* seed in ration at the alkaloid level of 1, 4, and 2.2 mg/kg/day for 4 days without any signs of toxicity being evident. However, the pigs did find the ration rather unpalatable. The latter observation was confirmed by Janssens and de Wilde (1989), who demonstrated decreased weight gains as a result of lower feed intake following exposure to linseed oil cake contaminated with *D. ferox* at a scopolamine level of 2 mg/kg.

It is also possible that the exposure to *Datura* induces neurological clinical signs. In a clinical case of poisoning, Spencer *et al.* (2000) reported head pushing against the pen bars in animals suspected to have been exposed to *D. stramonium*-contaminated maize; head pushing ceased on withdrawal of the feed. It was speculated that the clinical signs may have been due to hallucinations induced by scopolamine content of the plant. However, another plausible reason for the head pushing may be more behavioral than psychological effect. The muscarinic effect of the plant could easily have induced gastrointestinal stasis and subsequent colic, which would manifest as head pushing – a well-recognized sign of pain in animals.

The clinical and pathological changes induced by *Datura* are poorly documented for pigs. In a controlled study, pure scopolamine and atropine (98:2) were fed to 40 growing pigs at a concentration of 0, 1.5, 15, 75, and 150 mg/kg of feed for either 14 or 76 days (Piva *et al.*, 1997). Again, the major changes evident in all the treatment groups were decreased weight in comparison to the control. The two high doses also showed an increase in total plasma cholesterol, triglycerides, and total lipids, which the authors interpreted as increased fat mobilization. All animals also demonstrated an increase in plasma urea and uric acid, which suggests muscle



catabolism. The most prominent finding histologically was lymphocytic infiltration of the gastrointestinal tract mucosa with loss of epithelium and concurrent villus necrosis and dystrophic regeneration.

## Chickens

Similar to pigs, chickens are exposed to *Datura* due to the intensive diets they are fed. However, unlike pigs, chickens appear to be less susceptible to toxicity. In a study in which pure scopolamine and atropine (98:2) were included in a simulation of *D. ferox* exposure, five groups of broilers ( $N = 20$ ; 16 females and 4 males) were exposed to concentrations of 0, 1.5, 15, 75, and 150 mg/kg of feed, with no signs of muscarinic blockage. The only toxic effect was a reduction in weight gain for the higher dose group (Kovatsis *et al.*, 1993).

In a subacute study, Day and Dilworth (1984) fed milled *D. stramonium* seeds (unspecified toxic levels) to 1-day-old chicks at a concentration of 0, 1, 3, and 6% of the admixture of their diet. At 3 weeks, the two higher dose groups showed only lower weight gain. In a study by Flunker *et al.* (1987), milled *D. stramonium* was fed at 0.5 and 3% to six groups ( $n = 24$ ) of 1-day-old chicks for different time intervals or at concentrations of 1, 2, and 3% for 14 days to adult white leghorn hens ( $n = 5$ ). No deleterious effect on growth in the chicks or egg production was reported, with the exception of decreased feed intake (3%) in the high-dose hen group. In a subchronic study, Kovatsis *et al.* (1994) dosed pure scopolamine and atropine (98:2) at a concentration of 1.5, 15, 75, or 150 mg/kg to 100 egg-laying hens for 3 months. The high-dose group showed reduced egg production for the first 5 or 6 weeks, whereas lower doses had no effect. No changes in shell thickness, body weight, plasma aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, leucine aminopeptidase, and necropsy results were evident (Kovatsis *et al.*, 1994). As a result, it was concluded that broilers and layers could tolerate up to 3% of seeds in the diet for 14–21 days without adverse effects being evident. Long-term exposure, however, could decrease feed intake and interfere with performance (Damron, 1998).

## Other species

Cases of toxicities have been reported in dogs and cats following the accidental ingestion (through playing) of ornamental house plants. Unfortunately, the reporting publication did not mention the clinical signs seen in these animals (Berny *et al.*, 2010). A single case of a poodle dying has also been reported. The animal demonstrated

clinical signs of agitation, aggression, ambulatory delirium, tachycardia, tachypnea, mydriasis, and seizures that progressed to coma and death following exposure. At necropsy, the organs were congested, with lung edema, cardiac dilatation, and multiple pericardial petechiae; histopathology revealed hepatic necrosis, renal tubular degeneration, and widespread congestion and hemorrhage of the central nervous system (Tostes, 2002).

## TREATMENT

Treatment in most instances is supportive and symptomatic. Withdrawal of the contaminated feed or removal of animals from contaminated pastures should be the first step. For more severe cases, usually horses, more intensive management of the resulting colic is required. Treatment for the latter usually entails the infusion of crystalloid fluids, analgesic support, and, in cases of impaction, oral fluid administration and cathartics such as magnesium sulfate or mineral oil. In more severe cases, animals may require trocharization to relieve the gas distention and possibly even surgical intervention. The use of physostigmine as an antidote in animals appears to be ineffective, especially in horses presenting with clinical signs.

## CONCLUSIONS AND FUTURE DIRECTIONS

*Datura* poisoning is commonly reported in both people and animals. In the former, toxicity results mainly from the ingestion of the seeds, whereas in the latter, toxicity results from the contamination of the feed with either the plant or seeds. The clinical signs associated with toxicity are characteristic of the central and peripheral muscarinic inhibitory mechanism of the contained hyoscyamine and/or scopolamine toxins. Although the clinical signs are easily discernible, the clinical pathological and necropsy changes induced by the toxin are usually nonspecific. Toxicity is clearly species specific, with the susceptible domestic species being horses, pigs, cattle, and poultry. In addition, toxicity is related to the concentration of the toxic principles within the plant and the degree of adaptation of the animal. Treatment of toxicity is mainly supportive, with removal of the source of the toxin being imperative. Although the plant and its seed can pose major problems in animal production, these can be limited by proper feed management and screening.

## Ruminants

The available literature for cattle is scant and confusing to such an extent that some publications report cattle as being very susceptible, whereas others report them as being rather resistant. This suggests that rumen adaptation plays an important role in the development of toxicity. As such, it would be interesting to determine how much time is required for rumen adaptation and the degree of protection this offers to animals. It would also be interesting to ascertain if subclinical changes occur in these animals despite rumen adaptation.

## Toxicokinetics

Although a fair amount of literature is available on the toxicity of tropane alkaloids in animal species, there is a paucity of information on the general and interspecies toxicokinetics of the two toxins concerned. It would therefore be interesting to ascertain if the toxicokinetic parameters, such as absorption and metabolism, in the various domestic animals could be established. It would also be interesting to determine the importance of the first-pass effect in toxicity as well as the physiological protection offered by the blood-brain barrier of the different domestic species (i.e., whether atropine and scopolamine follow the same distribution patterns in all species). Another feature of toxicity that is poorly discussed is whether the release and absorption of the toxins is rapid (rapid and immediate release from the plant or a more slow and gradual release over time) and whether the liberated hyoscyamine isomerizes on absorption (i.e., whether studies that use pure hyoscyamine essentially only simulate 50% exposure to the toxic component of the plant).

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## Cottonseed toxicity

Steven S. Nicholson

### INTRODUCTION

Whole cottonseeds are a product of cotton (*Gossypium*) production. They are approximately 18% oil and 24% protein and contain polyphenolic pigments, one of which is the toxic component gossypol ( $C_{30}H_{30}O_8$ ; molecular weight, 518.55). The structural formula of gossypol is shown in Figure 86.1. Gossypol is a yellow liquid located within the pigment or oil glands in the cotton plant. In cottonseed, these glands appear as brown to black specks within the white matrix of the seed. The content of gossypol in whole cottonseed is free and unbound and ranges from 0.02 to 6.64%, and it is thought to provide resistance to insects (Price *et al.*, 1993). Free gossypol is not bound to protein and is available for absorption from the digestive tract in monogastric animals. In ruminants, some of the ingested free gossypol becomes bound to protein and is not absorbed. Gossypol is recognized for its toxic effect on cardiac, hepatic, pulmonary, and reproductive systems. Whole cottonseed, cottonseed meal, and cottonseed hulls are important feedstuffs. Variation of free gossypol levels in cottonseed meal is due to different processing methods used to extract oil as well as some natural variation in cotton varieties and an influence of growing factors (Cherry *et al.*, 1978). Cottonseed meal is a 36–41% protein supplement used in beef and dairy rations in the United States. Cottonseed hulls are used as a fiber source in cattle rations. The risk of gossypol toxicosis limits the amount of cottonseed meal in the diet of swine, poultry, dogs, and other monogastric animals as well as preruminant calves and preruminant lambs. The literature does not describe clinical cases in equids. Adverse effects of

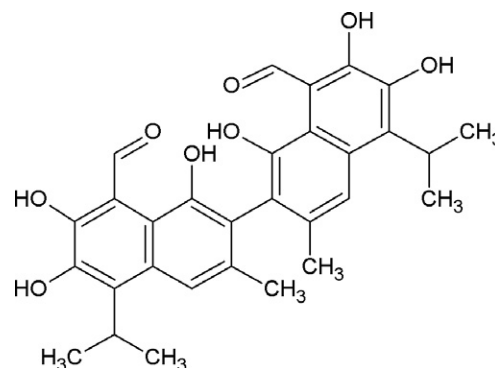


FIGURE 86.1 Structural formula of gossypol.

gossypol on reproduction in humans and animals may occur in certain situations.

### BACKGROUND

Free, unbound gossypol is one of several polyphenolic compounds found in the pigment glands of whole cottonseed. Cottonseed cake remains after oil is extracted from the seeds. Removal of the oil by commercial processors leaves cottonseed meal containing gossypol in two forms – free, which is the toxic form, and bound. Methods of extraction of the oil include mechanically extracted cottonseed meal containing 0.06% free gossypol and the expander solvent method cottonseed meal containing 0.14% free gossypol (NCPA, 2005). A solvent-only method of extraction produced cottonseed meal



with free gossypol content up to 0.6%. Bound gossypol is bound to amino acids in the meal and is considered unavailable for digestion. Some bound gossypol may be digested and absorbed by ruminants.

## PHARMACOKINETICS/ TOXICOKINETICS

Intake of free gossypol at a toxic level for a period of days or weeks by susceptible animals leads to the appearance of toxic effects. Gossypol tends to accumulate in the liver and kidneys. Little or no gossypol was detected in milk from cows fed cottonseed products (Lindsey *et al.*, 1980). The half-life of gossypol in three dairy cows ranged from 40.7 to 67.5 h (Lin *et al.*, 1991). Low dietary iron leads to increased gossypol absorption, and iron added to the diet can reduce absorption (Haschek *et al.*, 1989). Tissue concentrations in swine from highest to lowest were found in muscle, liver, fat, and red blood cells. In swine, gossypol is metabolized in the liver to various compounds, conjugated as glucuronides and sulfates, and excreted in the feces (Abou-Donia, 1989). In poultry, gossypol reacts with iron in egg yolks to produce an olive-green color. Cyclopropenoid fatty acids in cottonseed meal causes egg whites to become pink.

## MECHANISM OF ACTION

Gossypol seems to produce poisoning by more than one mechanism. Considerable evidence points to oxidative stress, formation of reactive oxygen species, and DNA scission – characteristics of redox cycling by electron transport in biosystems (Kovacic, 2003). Myocardial necrosis, congestive heart failure, and secondary hepatic changes and anemia due to complexing of gossypol with iron account for clinical signs and course of the toxicosis. Death may occur suddenly and unexpectedly in some cases due to cardiac failure.

## TOXICITY

Toxic levels of free gossypol in the diet produce cardiac necrosis and congestive heart failure in swine, dogs, goats, cattle, preruminant calves, and preruminant lambs. Dairy cows are susceptible to gossypol toxicosis.

Cottonseed products are seldom fed to horses. Horses appear to be fairly resistant to gossypol toxicity because

this problem has not been reported on a clinical basis in horses consuming cottonseed meal. However, because of problems with other species of livestock, possible gossypol toxicity should be noted as a concern. Precautions for feeding yearling horses cottonseed meal include the fact that it is low in lysine and to feed meal containing less than 0.2% gossypol. No effect on the health of young horses was reported when gossypol was fed to yearling horses at 115 ppm of the diet (Potter, 1981). Weanling-age and suckling foals showed no toxic effect at 348 ppm (McCall, 1982).

High mortality was seen in dairy calves fed cottonseed meal with 250–380 ppm gossypol during the first 10 weeks of life (Holmberg *et al.*, 1988). Holstein calves may develop ascites, diarrhea, and weakness after consuming toxic levels of gossypol in a diet containing cottonseed meal for 70 to approximately 180 days. In the author's experience (unpublished data), a typical case of gossypol poisoning presents as follows. Holstein calves 3–8 months of age were confined to dry lot and fed up to 3.6–4.5 kg per day of lactating cow ration for 4 months with little or no hay available. The feed contained 600 lb (272 kg) cottonseed meal per ton. The free gossypol content of the cottonseed meal was determined to be 0.6%. Two calves died approximately 1 day apart from what was thought to be pneumonia because of cough, dyspnea, and blood-tinged nasal discharge. Two calves were sick at the time of the farm visit. One had diarrhea, ascites, dyspnea, anemia, and weakness. At necropsy, ascites was marked and the liver was swollen, the intestinal tract was edematous, the heart had pale areas within the myocardium that were later confirmed as areas of necrosis, and the lungs were edematous. Other causes of myocardial necrosis, including the plant oleander (*Nerium oleander*), ionophores, excess selenium injection, *Senna* (formerly *Cassia*) myopathy, and white muscle disease, were ruled out. In a published incident, 24 of 57 calves fed a diet containing 33% cottonseed meal died at between 7 and 15 weeks of age. Initial deaths were not accompanied by premonitory signs, but after cottonseed meal withdrawal, most calves developed rough coats, anorexia, weakness, ascites, and subcutaneous edema. Those that died had large volumes of serous fluid in the body cavity, firm liver of "nutmeg" appearance, and pulmonary congestion. Histopathology revealed periportal hepatic necrosis in acute cases and periportal fibrosis in chronic cases. Lungs from several calves had edema, hemosiderosis, and fibrosis in some pulmonary vessels. Atrophy of myocardial fibers was present in most cases. The concentration of free gossypol in the diet was 100–220 ppm. Antemortem and postmortem findings supported a diagnosis of gossypol poisoning. The deaths continued for 4 weeks after withdrawal of cottonseed meal from the diet (Zelski *et al.*, 1995).

Gossypol poisoning in mature cattle is uncommon, but dairy cows can be at risk because they may be fed both

cottonseed and cottonseed meal during lactation. Dairy cows consuming high levels of cottonseed meal may be at risk of cardiac failure (Jimenez, 1979; Lindsey *et al.*, 1980; Smalley and Bicknell, 1982). High environmental temperature and humidity seem to enhance the risk. Dairy cows tolerated up to 6.2mg/kg body weight free gossypol per day for 100 days, but 8.8mg/kg caused red cell fragility, depressed hemoglobin levels, and increased respiratory rate in response to elevated ambient temperatures (Lindsey *et al.*, 1980). Erythrocyte fragility on days 28 and 56 and elevated serum alkaline phosphatase were noted, but adverse reproductive effects were not seen in crossbred heifers fed a diet of 30% cottonseed (Calhoun *et al.*, 1991). Consumption of a high-gossypol diet for 170 days had no effect on the health of lactating dairy cows, but it increased plasma gossypol concentrations and impaired reproductive performance (Santos *et al.*, 2003). Ammoniation of cottonseed meal to reduce aflatoxin content was thought to have enhanced the toxic effects of gossypol in dairy cattle fed 6–9lb of cottonseed meal per day (Smalley and Bicknell, 1982).

Rogers and Moore (1995) reported that it was safe to feed higher levels of free gossypol in the diet when the source of free gossypol was whole cottonseed instead of cottonseed meal. For example, for mature cows and bulls, the recommended safe levels of free gossypol in the diet were 900 and 1200ppm, respectively, when whole cottonseed was fed compared with 200 and 600ppm, respectively, when the source of free gossypol was cottonseed meal. The higher levels of free gossypol recommended for diets containing cottonseed reflect the fact that free gossypol in unprocessed whole cottonseed is extensively bound during digestion in the rumen and is less available than free gossypol in cottonseed meal (Calhoun, 1995).

Lambs fed a diet containing 900ppm free gossypol for 3 or 4 weeks died suddenly or after exhibiting signs of cardiac failure (Morgan *et al.*, 1988). Clinical signs included sudden death and/or chronic dyspnea syndromes. Serum chemistry alterations in the lambs included high serum total lactate dehydrogenase and lactate dehydrogenase liver-specific isoenzyme activities. Serum total creatine kinase activity decreased markedly in lambs of all groups treated with gossypol at 45, 136, or 409mg per day. All lambs given 409mg of gossypol per day died before the end of the 30-day study.

Gossypol in the diet at 250–300mg of free gossypol per day for several months apparently caused congestive heart failure in adult goats (East *et al.*, 1989).

In swine, dietary levels of 200–300ppm free gossypol result in cardiac insufficiency and respiratory distress with mortality rates exceeding 50% (Haschek *et al.*, 1989). A level of 100ppm free gossypol in the diet of swine is considered safe.

In dogs, cardiotoxicity associated with prolonged gossypol intake is similar to that in other species. Four

dogs died, with lesions compatible with those described in experimental gossypol poisoning, after eating food sprinkled with cottonseed meal over a period of months. The amount of free gossypol fed was approximately 5.4–5.7mg/kg body weight per day for an unknown duration (Patton *et al.*, 1985). Six dogs died after accidental ingestion of cottonseed bedding, but clinical signs of illness were not seen. At necropsy, the lungs were congested and edematous, and the liver was firm, congested, and had a marked reticular pattern. Moderate ascites was noted. Histopathology revealed multifocal myocardial degeneration and necrosis, severe pulmonary edema, and chronic passive congestion of the lungs, heart, liver, and kidneys. Transmission electron microscopy of the myocardium revealed disruption of myofibrils, chromatin condensation, and disrupted and swollen mitochondria. The cottonseed bedding contained 1600mg/kg of free gossypol, a concentration considered toxic for monogastric animals. The stomach content revealed the presence of gossypol. Gossypol poisoning in dogs is extremely rare and had not previously been associated with cottonseed bedding (Uzal *et al.*, 2005).

Rabbits and guinea pigs are susceptible to gossypol toxicosis.

Severe cases of perivascular lymphoid aggregate formation, biliary hyperplasia, and hepatic cholestasis were observed in chicks fed 800 and 1600mg/kg of purified gossypol in feed (Henry *et al.*, 2001b). Gossypol was added to broiler feed at 0, 100, 200, and 400mg/kg of feed in experiment 1 and at 0, 800, and 1600mg/kg of feed in experiment 2. Feed conversion ratios of chicks fed 800 and 1600mg/kg gossypol were significantly higher than those of chicks fed control diets (1.383 vs. 1.564 vs. 1.745 for 0, 800, and 1600mg/kg gossypol, respectively). Enlarged gallbladder was the only gross pathologic lesion associated with gossypol levels. Severe cases of perivascular lymphoid aggregate formation, biliary hyperplasia, and hepatic cholestasis were observed in chicks fed 800 and 1600mg/kg of gossypol in feed. No gossypol-related changes were observed in kidney tissues of chicks. These results show that gossypol is toxic to broiler chicks at high levels. This study also shows that histopathology changes in liver due to gossypol also occur at levels lower than those that affect body weight (Henry *et al.*, 2001b). In another experiment, broiler chicks were fed diets containing 20% feed-grade or extruded cottonseed meal and compared with control chicks fed corn and soybean meal-based broiler rations. Weight gain, feed intake, and feed conversion ratio of broilers at 21 days were significantly affected by the diets. Feeding feed-grade and extruded cottonseed meal resulted in decreased body weight gain, increased feed intake, and inefficient feed utilization. When 2% lysine was added to feed-grade or extruded meal, body weight gains of chicks were not significantly

## TREATMENT

different from those fed the control diet. The extrusion process reduced the free gossypol in the meal, but the total gossypol level was not changed and chick performance was not improved. The authors concluded that with adequate supplemental lysine, cottonseed meal can be used in broiler diets without a reduction in performance (Henry *et al.*, 2001a).

In fish, the pathological effects of gossypol in tilapia (*Oreochromis* sp.) were a decline in body weight, low hemoglobin and hematocrit levels, an abundance of immature red blood cells, and abnormal spleen morphology (Garcia-Abiado *et al.*, 2004). In rainbow trout, feeding diets containing cottonseed meal during a period of 10 months did not result in differences in growth and mortality compared to the control group ( $P > 0.05$ ). Gossypol was transferred to the eggs, and embryo survival increased linearly as gossypol content in the diet was increased (Blom *et al.*, 2001). In channel catfish fed cottonseed meal, there was no toxic effect of free gossypol noted at 900 ppm (Dorsa *et al.*, 1982).

In summary, the signs and lesions of gossypol toxicosis include myocardial necrosis, congestive heart failure, centrilobular necrosis of the liver, pulmonary edema, and perhaps mild hemoglobinuria caused by red blood cell fragility. Mild to moderate anemia may be present. Ascites, diarrhea, and edema of the intestinal tract secondary to congestive heart failure may be seen. Dairy cows and lambs may die suddenly with minimal lesions. Death caused by heart failure may occur several weeks after last consumption of toxic levels of gossypol.

The diagnosis of toxicosis includes history of prolonged dietary exposure, clinical signs and lesions compatible with acute or chronic heart failure, and elimination of other potential causes.

Adverse male reproductive effects are of concern because studies have found a decrease in testicular steroidogenesis and spermatogenesis in pubescent rams and bulls (Lin *et al.*, 1981; Kramer *et al.*, 1991). Reversible male infertility has been reported for some animal species and humans (Abou-Donia, 1989). In peripubertal bulls, gossypol (8 mg/kg per day for 56 days) increased sperm abnormalities. The number of sperm with proximal droplets was significantly higher in the gossypol-treated bulls, suggesting testicular degeneration, but the effects were reversible (Hassan *et al.*, 2004). Extensive damage to the germinal epithelium has been shown in both rams and bulls that were fed diets containing gossypol, and this is of concern (Randel *et al.* 1992). Its use in the diet of prepubertal bulls may be a concern. However, cottonseed meal has been and remains an important winter feeding supplement for beef cattle herds in the southwestern United States. The generally cited recommendation for limiting cottonseed meal in the diet for young developing bulls is based on a maximum of 150 ppm free gossypol in the diet.

Gossypol sources in the diet should be removed. Treatment of food animals affected by gossypol toxicosis is impractical. Adding ferrous sulfate to the diet has a protective effect. Generally, ferrous sulfate added to the diet in amounts equal to free gossypol inactivates gossypol and thereby reduces its toxicity (Barraza *et al.*, 1991). Feed supplemented with high protein levels can also provide a protective effect by reducing free gossypol. Dogs may benefit from therapy designed to strengthen heart function in congestive heart failure.

## CONCLUDING REMARKS

Pelleting reduced free gossypol by as much as 70% in whole cottonseed and by 48% in cottonseed meal (Barraza *et al.*, 1991). Pelleting represents a mechanism to decrease the toxicity of gossypol in cottonseed. Preruminant calves, early weaned lambs, pigs, and poultry can safely be fed diets containing up to 100 ppm free gossypol without fear of gossypol toxicity.

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## Fescue toxicosis

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### INTRODUCTION

“Fescue toxicosis” is one of the most economically costly grass-related intoxications of livestock in the United States, as well as other regions of the world. Historically, there were portions of the United States where particularly unpredictable weather conditions resulted in less than desirable pasture development for grazing livestock. A hardy tall fescue grass, which has undergone a series of changes in nomenclature during the past 40 years (currently *Lolium arundinaceum* [Shreb.] Darbysh., *Schedonorus arundinaceus* [Schreb.] Dumort., or *Schedonorus phoenix* [Scop.] Holub; formerly *Festuca arundinacea* Schreb. var. *arundinacea* Schreb. or *Festuca elatior* L.), was obtained from a Kentucky farm in 1931 and was first marketed commercially in 1943 (Ball, 1984; Burrows and Tyrl, 2001; Strickland *et al.*, 2011). Today, this variety of tall fescue, Kentucky 31, occupies more than 20 million ha in the United States and represents an important grazing grass, especially within the southeastern and southern mid-western regions of the United States (Evans *et al.*, 2004b; Stuedemann and Seman, 2005), as well as internationally. It is a cool-season grass that grows well in the fall, producing substantial forage for the winter months. By 1948, a disease syndrome referred to as “fescue foot” had been described in New Zealand, and in 1950, the same syndrome was reported in the United States (Bacon, 1995). In 1973, it was noticed that cattle on a Kentucky 31 pasture were very unthrifty compared to cattle on an adjoining nonfescue pasture (Schmidt and Osborn, 1993). This observation initiated an investigation that revealed a fungus growing within the plant (endo = within; phyte = plant) (Bacon *et al.*, 1977). Today, it is believed that

greater than 95% of tall fescue pastures containing mostly the Kentucky 31 variety are infected by the endophyte, *Neotyphodium coenophialum* (Yates and Powell, 1988). Endophyte-infected (E+) fescue is estimated to cause more than \$1 billion in lost livestock production in the United States each year (Oliver, 1997).

### BACKGROUND

#### Endophyte name

Endophytes are not uncommon in a number of forage grasses and other pasture plants, and unlike other fungal infections, such as those associated with species of *Claviceps*, they are not visible to the naked eye because they grow “within” the plant (Evans *et al.*, 2004a, 2004b). Bacon *et al.* (1977) initially discovered the endophyte and based on earlier morphologic research classified it as *Epichloë typhina*. This endophyte was later renamed *Acremonium coenophialum* (Morgan-Jones and Gams, 1982). Further molecular phylogenetic evaluation has generated the latest name for the tall fescue endophyte, *N. coenophialum* (Morgan-Jones and Gams, 1982; Glenn *et al.*, 1996), and a similar endophyte, *Neotyphodium lolii*, is found in perennial ryegrass.

#### Mutualism (symbiosis) and endophyte survival

The tall fescue plant and its endophyte enjoy a mutualistic or symbiotic relationship (Thompson *et al.*, 2001). Each benefits from the survival of the other. The plant

supplies a comforting and safe internal environment for the endophyte along with all the required nutrients. In return, the endophyte lives in intercellular spaces in the plant without disrupting cells of the plant. The endophyte generates multiple toxins that are distributed throughout the plant. The toxins make the plant more resistant to drought, insects, parasitic nematodes, fungi, and herbivores. The endophyte invades seed heads of the plant and is able to continue its relationship with the next generation of fescue through its contamination of the seed. Unlike ergotized (*Claviceps purpurea*-infected) tall fescue, endophyte infections cannot be transferred naturally to a non-endophyte-infected variety of tall fescue (Evans *et al.*, 2004a). However, E+ varieties of fescue are more hardy and persistent. They produce more forage and seeds than non-endophyte fescue plants and, thereby, may eventually take over a pasture.

## Endophytic toxins

Multiple classes of toxins are produced by *N. coenophialum*, including ergot alkaloids, loline alkaloids, and peramine (Porter, 1995). Loline alkaloids and peramine are primarily insect deterrents. Loline alkaloids belong to an aminopyrrolizidine group of alkaloids but are not known to cause hepatic problems similar to the pyrrolizidine alkaloids in other plant species. The loline alkaloids found in fescue include loline, *N*-acetyl loline, *N*-formyl loline, *N*-acetyl norloline, and *N*-methyl loline (Porter, 1994). Several of these compounds, particularly *N*-acetyl norloline, have been associated with equine fescue edema syndrome involving a genetically modified form of *N. coenophialum* in the Mediterranean variety of tall fescue (Bourke *et al.*, 2009).

## Ergot alkaloids

### General classification and toxicity

The ergot alkaloids are a large class of compounds, for which the nomenclature can sometimes be confusing. Ergot alkaloids found in fescue include the lysergic acid amides (e.g., ergonovine), sometimes also referred to as ergoline alkaloids; the clavine alkaloids; and the peptide or peptine alkaloids (Porter, 1995; Evans *et al.*, 2004b; Strickland *et al.*, 2011). Ergovaline is a peptide or ergopeptide (i.e., peptine or ergopeptine) alkaloid with a lysergic acid structure combined with three amino acids. It differs in structure from ergotamine by one amino acid. Among the ergot alkaloids found in tall fescue, ergovaline is by far the most prevalent ergopeptide (Porter, 1995). It accounts for approximately 90% of the ergopeptide alkaloid content of tall fescue (Lyons *et al.*, 1986). The

concentrations of ergovaline in the stems and leaves of tall fescue usually range between 200 and 600 ppb and in the seed heads from 2000 to 4000 ppb (Evans *et al.*, 2004b). Estimates of the minimum threshold toxic concentrations of ergovaline in fescue, which have the potential to cause clinical problems in livestock, range from 50 to 500 ppb (Moubarak *et al.*, 1993; Tor-Agbidye *et al.*, 2001; Aldrich-Markham *et al.*, 2003; Evans *et al.*, 2004b). These minimal threshold concentrations depend on the species affected, the type of syndrome being considered, and environmental temperatures, and they will most likely vary with geographical location. Species susceptibility from most to least susceptible is as follows: horses (i.e., late-gestational mares) > cattle > sheep > camelids (Aldrich-Markham *et al.*, 2003; Evans *et al.*, 2004b). In general, total dietary ergovaline concentrations greater than 100–200 ppb on a dry weight basis are potentially toxic for cattle and horses, depending on other contributing factors (Rottinghaus *et al.*, 1991; Blodgett, 2001; Evans *et al.*, 2004b). The clinical signs observed following animal exposure to ergopeptide alkaloids are dependent on animal species and physiological state, environmental conditions, relative toxicities of and interactions between different ergopeptide alkaloids, and the level as well as duration of ergopeptide alkaloid exposure (Evans *et al.*, 2004b). For example, dietary concentrations of ergovaline as low as 50 ppb have been associated with clinical cases of agalactia in mares.

### Role of ergovaline in fescue toxicosis

The ergopeptide alkaloids are believed by many to be the major toxins responsible for the multiple fescue toxicosis syndromes observed in animal species (Porter and Thompson, 1992; Evans *et al.*, 2004b). Because ergovaline is the most prominent ergopeptide alkaloid, it probably either dictates the likelihood of a toxicosis occurring or, at least, is a reliable biomarker for toxicity. Ergovaline has traditionally been difficult to purify and synthesize, and therefore little research has been done with pure ergovaline to prove that it is the primary toxin in fescue. Although subject to some debate, there are multiple reasons to believe that ergovaline is the primary toxin or, minimally, at least serves as a biomarker for the content of ultimate toxicant in E+ fescue. An experiment with synthetic ergovaline in sheep reproduced most clinical signs of the summer slump syndrome seen in cattle (Gadberry *et al.*, 2003). Ergovaline injected intraperitoneally into cattle for 3 days produced increased rectal temperatures and respiratory rates observed with summer slump (Spiers *et al.*, 2005a,b). Other ergopeptide alkaloids, including ergotamine and bromocryptine, are also able to reproduce fescue toxicosis syndromes (Evans *et al.*, 1999, 2004b; Blodgett, 2001). Ergovaline is a very potent vasoconstrictor in *in vitro* models (Oliver *et al.*, 1998). In addition, ergovaline is also a strong prolactin

inhibitor, whereas ergonovine, a lysergic acid amide in E+ fescue, is unable to produce typical signs of fescue toxicosis or lower prolactin concentrations in cattle (Oliver *et al.*, 1994). Antibodies against ergot alkaloids, including – to some extent – the ergopeptine alkaloids, are able to ameliorate some clinical features of fescue toxicosis in cattle and mice (Hill *et al.*, 1994; Rice *et al.*, 1998). New, novel endophyte varieties of fescue that lack ergovaline, but not other types of fescue endophytic toxins, do not cause fescue toxicosis (Roberts and Andrae, 2004). Syndromes similar to fescue toxicosis are seen when ergotized grains infected by *C. purpurea* are ingested by livestock (Thompson *et al.*, 2001; Evans *et al.*, 2004a). Finally, it has been shown that D<sub>2</sub> dopamine antagonists, which stimulate prolactin secretion, are able to alleviate most of the toxic effects of fescue (Lipham *et al.*, 1989; Cross, 1997; Evans *et al.*, 1999). With improved analytical capabilities, it might be possible in the future to discern more accurately the exact roles of specific ergot alkaloids and their various isomers in the pathogenesis of fescue toxicosis (Strickland *et al.*, 2011).

#### Seasonal variation

The concentration of ergot alkaloids in fescue pastures varies with the season, with ergovaline concentrations reported to be low in the spring (300–500ppb) and reaching peak concentrations in seed heads (up to 1000–5000ppb) during the summer months (Rottinghaus *et al.*, 1991). Concentrations of ergovaline decrease somewhat during the early fall and rebound with fall regrowth. Seed heads consistently contain the highest concentration of ergovaline. Stockpiled fescue pasture has more ergot alkaloids early in the winter than late in the winter (Roberts and Andrae, 2004). Drought and rainy conditions tend to increase ergovaline concentrations (Arechavaleta *et al.*, 1992; Aldrich-Markham *et al.*, 2003). Fertilization of fescue pastures with nitrogen- and phosphorus-based fertilizers or poultry litter also increases ergot alkaloid concentrations (Stuedemann and Seman, 2005). Because ergovaline is a mycotoxin and mycotoxin production and persistence are dependent on multiple seasonal and environmental conditions, it is not surprising that concentrations of ergovaline in tall fescue would be expected to vary from season to season and year to year (Lyons *et al.*, 1986).

#### Interactions with *Claviceps purpurea*

Although ergotism in livestock is sometimes considered a historical problem, instances of clinical disease associated with the ingestion of ergotized small grains or pasture grasses, including E+ and non-endophyte-infected tall fescue, continue to occur in the United States (Evans,

*et al.*, 2004a,b). This is especially true when grain or grass screenings (fines) are used in feedstuffs. Because of the much higher concentrations of ergopeptine alkaloids present in ergot sclerotia, compared to endophytic mycelia, it is important to evaluate the potential for ergotism in instances in which the typical clinical signs of fescue toxicosis appear to be less dependent on environmental temperature or there is no history or evidence of exposure to E+ fescue (Evans *et al.*, 2004a).

## PHARMACOKINETICS/ TOXICOKINETICS

### Absorption

Although relatively little research has been devoted to *in vivo* absorption of ergot alkaloids in livestock species, a growing body of literature involving the pharmacokinetics/toxicokinetics of ergot alkaloids provides at least some additional insight into how ergot alkaloids are handled by the body following oral exposure (Strickland *et al.*, 2011). The rumen and small intestine are most likely the principal sites of ergot alkaloid uptake, which, depending on pH, animal species, and physiological state, can involve both passive and active processes. After cannulation of ruminal, gastric, and mesenteric veins of sheep consuming E+ fescue, ergot alkaloids were detected only in ruminal veins (Hill, 2005). However, *in vitro* tissue specimens of rumen, reticulum, and omasum are all capable of ergot alkaloid transport. It is currently thought that most of the gastrointestinal absorption of the ergopeptine alkaloids takes place in the small intestine (Strickland *et al.*, 2011). Ergovaline is difficult to detect in rumen fluid and is metabolized by rumen microbes to lysergic acid. Other researchers have found that although 50–60% of ingested ergovaline is in the abomasal contents, minimal amounts of ergovaline remain in the ileal contents or feces (Oliver, 1997).

### Distribution/metabolism

Ergovaline is present only in parts per billion or low parts per million concentrations in E+ tall fescue, so the dilution factor in the body and sensitivity of various methods make evaluation of the concentration in any organ extremely difficult. Therefore, relatively little is known about the distribution of ergovaline and other ergot alkaloids in the body. Intravenous injection of several ergopeptides in calves documented a distribution and tissue equilibrium phase in serum lasting approximately 1 h (Moubarak *et al.*, 1996). This was followed by

an elimination phase with a half-life of approximately 20–30 min. It is known that multiple systems in the body may show some effects of ergot alkaloid consumption, including the cardiovascular system, central nervous system, and abdominal fat. There is evidence that ergopeptine alkaloids accumulate in adipose tissue, but experimental results have not been entirely consistent (Strickland *et al.*, 2011). In ruminants, ergovaline undergoes microbial breakdown to lysergic acid and related compounds. Ergovaline is believed to be metabolized in the liver by the cytochrome P450 3A subfamily of enzymes (Moubarak and Rosenkrans, 2000; Moubarak *et al.*, 2003; Settivari *et al.*, 2008; Strickland *et al.*, 2011). Cytochrome P450 3A4 is responsible for adding one or two hydroxyl groups to the peptide ergoline ring to make it more hydrophilic for excretion (Moubarak and Rosenkrans, 2000). Interestingly, this enzyme family is inducible in rats treated with dexamethasone but is not induced in rats with prior ergot alkaloid exposure (Moubarak *et al.*, 2003). However, CYP450 induction has been shown in *in vitro* studies (Settivari *et al.*, 2008), and sheep fed E+ fescue had increased hepatic activity of mixed function oxidases (Zanzalari *et al.*, 1989). The indole ring of ergopeptine alkaloids may also undergo eventual oxidation (Moubarak and Rosenkrans, 2000). Whether or not other groups of enzymes are involved in metabolizing or conjugating ergovaline is not known.

## Excretion

Urine, bile, feces, and, to a much lesser extent, milk are the primary routes of elimination and excretion for ergot alkaloids (Strickland *et al.*, 2011). Metabolites of ergot alkaloids have been measured indirectly with lysergol antibodies in bile and urine of cattle (Stuedemann *et al.*, 1998). Ninety-six percent of the metabolites in cattle are found in urine. Ergot alkaloids are detected in urine within 12 h of exposure to E+ fescue and are maximal within 24 h. After removal from a fescue pasture, ergot metabolites are gone within 48 h from the urine of cattle. Similarly, when removed from pastures containing E+ fescue, post-term mares may show signs of mammary development and impending parturition within 48 h of removal from pasture (Schmidt and Osborn, 1993).

## MECHANISMS OF ACTION

### D<sub>1</sub> dopaminergic antagonist

Activation of D<sub>1</sub> dopamine receptors has been reported to be associated with vasodilation, and antagonism of

these receptors has been reported in conjunction with ergot alkaloids (Cross *et al.*, 1995). The precise role of this mechanism in the gangrene of the extremities described later has not been determined.

### D<sub>2</sub> dopaminergic agonist

#### *Prolactin inhibition*

Ergovaline is a dopamine D<sub>2</sub> receptor agonist (Oliver, 1997). Ergopeptine alkaloids have a 10-fold greater affinity for dopamine D<sub>2</sub> receptor binding than do ergoline alkaloids (Larson, 1997). Dopamine agonists mimic the endogenous tonic inhibition of pituitary lactotropes by dopamine and inhibit prolactin secretion by the anterior pituitary. Prolactin inhibition is one of the most consistent problems observed in multiple species exposed experimentally to ergopeptine alkaloids and/or experiencing clinical fescue toxicosis. In essence, this effect of ergovaline and other ergot alkaloids is an exquisite, naturally occurring example of “endocrine disruption” and is a sensitive biomarker for exposure to these compounds.

#### *Lactation suppression*

One of the roles of prolactin is induction of mammary gland growth and milk production (i.e., lactogenesis). Hypoprolactinemia is often associated with agalactia at parturition in many species, especially the horse and pig. Ruminant species have a placental lactogen that can overcome this initial lack of prolactin stimulation of the mammary gland prior to and at birth (Cross, 1997). However, lower prolactin concentrations in dairy and beef cows consuming E+ fescue, as well as diminished feed intake associated with the ingestion of E+ fescue, can be reflected by decreased milk production (up to 50%) observed after the perinatal period (Schmidt and Osborn, 1993; Thompson *et al.*, 2001; Evans *et al.*, 2004b).

#### *Effect on other reproductive hormones*

Prolactin facilitates corpus luteal function and gonadotropin secretion (Porter and Thompson, 1992). Altered luteal function in heifers grazing E+ fescue can result in reduced progesterone. Consumption of E+ fescue and ergopeptide exposure have been associated with low progesterone production in both cows and horses, diminished progestagens in late-gestational mares (5 $\alpha$ -pregnanes rather than progesterone normally predominate the last two trimesters of equine pregnancy), suppressed relaxin associated with possible impaired placental function, and, somewhat more variable, alterations in estrogen concentrations in pregnant mares (Cross, 1997; Evans *et al.*, 1999; Thompson *et al.*, 2001; Evans, 2011). These imbalances of reproductive



hormones lead to early pregnancy problems in cattle and late pregnancy problems in horses. Ergopeptide potency for prolactin inhibition has been correlated with inhibition of ovum implantation in rats (Fluckiger *et al.*, 1976). Ergovaline is intermediate among the ergopeptides in its ability to inhibit implantation, but it is approximately twofold more potent than ergotamine.

#### *Effect on hypothalamic thermoregulatory center*

Another role of dopamine and/or prolactin is control of the thermoregulatory center in the hypothalamus (Strickland *et al.*, 1993). Diminished prolactin and/or dopamine receptor perturbation causes the thermoregulatory center to deregulate and contributes to the development of hyperthermia or hypothermia observed in exposed animals. Deregulation is more likely when environmental temperatures are outside of the thermoneutral range of the animal (Spiers *et al.*, 2005a,b). Fescue foot problems are more likely at temperatures less than 8°C (Tor-Agbidye *et al.*, 2001), whereas summer slump problems are more apparent when temperatures exceed 31°C (Schmidt and Osborn, 1993; Spiers *et al.*, 2005a,b).

#### *Effect on lipogenesis*

Prolactin also has a role in lipogenesis through control of metabolism of cholesterol and triglycerides by the liver (Strickland *et al.*, 1993). Cattle suffering from summer slump traditionally have low serum cholesterol and triglyceride concentrations (Oliver, 1997). Low serum cholesterol is also commonly found in cattle herds with abdominal fat necrosis (Schmidt and Osborn, 1993). Necrotic abdominal fat is lower in ether-extractable material, but it has a much higher cholesterol content of the ether-extractable fraction. Lipolysis in cattle experiencing fescue toxicosis is decreased (Thompson *et al.*, 2001). Metoclopramide, a dopamine antagonist and prolactin enhancer, increases serum cholesterol levels in steers grazing E+ fescue pastures (Lipham *et al.*, 1989). Serum triglycerides and cholesterol are also reduced by  $\alpha_1$  adrenergic receptor antagonists and  $\alpha_2$  adrenergic agonists, which are both physiologic effects of ergot alkaloids (Oliver, 1997).

#### *Effect on winter hair loss*

Change in photoperiod with the end of winter and the start of spring normally will increase prolactin production and stimulate shedding of long winter hair in favor of new spring hair coat growth (Thompson *et al.*, 2001). Associated with a decrease in prolactin, cattle experiencing fescue toxicosis often fail to shed some or all of their long winter hair. This long hair may become bleached out by the summer sun and contributes to an unthrifty appearance. In addition, fescue toxicosis has

been associated with alterations in copper homeostasis (probably associated with decreased feed intake), which most likely also contributes to the discolored appearance of the hair coat in animals on E+ fescue pasture.

#### *Effect on immunity*

Prolactin is considered an immunomodulator (Strickland *et al.*, 1993). Impaired immune function from E+ fescue seed was noted in mice and rats, whereas cattle were not affected. However, numerous other E+ fescue studies in cattle cite decreased antibody response, reduced globulins, or increased morbidity and mortality (Thompson *et al.*, 2001). In contrast, Rice *et al.* (1997) noted increased humoral immunity in cattle fed E+ fescue.

#### *Miscellaneous neurologic effects*

Other miscellaneous neurologic effects include a consistently negative effect of dopamine agonists on feed intake, which can have a major impact on consumption of both macro- and micronutrients. Ergopeptide alkaloids may also interfere with gastrointestinal motility, and there evidence that at least motility of the reticulo-rumen compartment is inhibited by these compounds (Strickland *et al.*, 2011). Use of a dopamine antagonist, metoclopramide, increased feed intake in lambs fed E+ fescue without an effect on body temperature (Thompson *et al.*, 2001). Nervous behavior in cattle fed E+ fescue might be related to the ability of ergopeptide alkaloids to release dopamine in *in vitro* synaptosomal preparations (Larson, 1997).

### $\alpha_1$ Adrenergic antagonist

Ergopeptide alkaloids are  $\alpha_1$  adrenergic receptor antagonists as well as  $\alpha_2$  receptor agonists (Oliver, 1997). Many of the most characteristic clinical effects of ergopeptide alkaloids are easily described in terms of  $\alpha_2$  receptor agonism (discussed later). The assessment of the net effects of interactions between ergot alkaloids and adrenergic receptors in the animal requires that the antagonistic effects of these compounds on  $\alpha_1$  receptors be interpreted in light of the endophytic toxins' agonistic effects on  $\alpha_2$  receptors.

### $\alpha_2$ Adrenergic agonist

#### *Vasoconstriction*

#### *Gangrene of extremities*

Ergovaline acts as a potent  $\alpha_2$  adrenergic agonist on blood vessels, especially arterioles (Oliver, 1997). The persistent vasoconstriction of peripheral arterioles in

the back legs of cattle consuming fescue is believed to be responsible for thickening of the smooth muscle wall of the arterioles seen with fescue foot problems (Thompson *et al.*, 2001; Strickland *et al.*, 2011). Chronic exposure of cattle to E+ fescue makes their  $\alpha_2$  adrenergic receptors more reactive to ergot alkaloids (Oliver, 1997).

#### *Decreased heat loss*

Constriction of blood vessels in the skin of cattle also contributes to hyperthermia during the summer months. In addition to the thermoregulatory effects of dopamine/prolactin on the hypothalamus, the decreased dissipation of heat through the skin of cattle is believed to contribute to the higher body temperature observed when summer temperatures are at or above 30°C (Thompson *et al.*, 2001).

#### *Serum enzyme decrease*

Multiple serum enzyme decreases in cattle have been associated with inhibition of adenylyl cyclase levels due to  $\alpha_2$  adrenergic activity of ergovaline (Oliver, 1997). Ergovaline inhibits cyclic AMP production via the  $\alpha_{2a}$  adrenergic receptor (Larson, 1997). In addition to the aforementioned decrease in serum cholesterol and triglycerides, there are also decreases in alkaline phosphatase,  $\gamma$ -glutamyltransferase, aspartate aminotransferase, alanine aminotransferase, creatinine kinase, lipase, and lactic dehydrogenase (Oliver, 1997). Potentially, the inhibition of ATPase in brain and kidney may be mediated by the same mechanism (Moubarak *et al.*, 1993).

#### *Oxidative stress*

A few studies have noted oxidative stress effects in cattle grazing E+ fescue pastures (Oliver, 1997).  $\alpha_2$  Adrenergic receptor agonists have the capability of depleting glutathione, a peptide integral in defending against oxidative stressors. Lakritz *et al.* (2002) noted reduced glutathione concentrations in whole blood samples from cattle exposed to heat stress and E+ fescue. Settivari *et al.* (2008) observed decreases in antioxidant proteins following exposure to endophytic toxins.

#### *Renal-related effects*

$\alpha_2$  Adrenergic receptor agonists decrease antidiuretic hormone release by the pituitary (Oliver, 1997). This causes an inability of cattle to concentrate their urine. Ergot alkaloids also block aldosterone production in the adrenals (Oliver, 1997). Decreased aldosterone and antidiuretic hormone promote diuresis, and cattle coming to feedlots from E+ fescue pastures have a reputation for producing a muddy wallow in their pens. Aldosterone action could also be compromised by lowered kidney

sodium/potassium-ATPase activity seen with ergot alkaloid exposure (Oliver, 1997).

### **Serotonergic agonist**

Ergot alkaloids also act on serotonergic<sub>2</sub> receptors (Oliver, 2005). Ergovaline is an agonist at serotonergic<sub>2</sub> receptors of uterine and umbilical arteries (Dyer, 1993). This serotonergic activity in blood vessels causes persistent vasoconstriction *in vitro*. Serotonergic activity of ergot alkaloids may also be important in the enhanced mitogenesis of vascular smooth muscle, hypothalamic thermoregulatory center effects, pulmonary vasoconstriction and bronchoconstriction, and, importantly, the appetite suppression seen with fescue toxicosis (Oliver, 1997, 2005).

## **TOXICITY**

### **Seasonal variation and effects of decreased feed intake**

#### *Cattle*

##### *Summer slump*

Summer slump or summer syndrome is the most common and costly syndrome seen in cattle with fescue toxicosis. Although it is most dramatic during the summer when environmental temperatures reach above 31°C, the problem has been reported to occur during other times of the year as well, but the possibility of concurrent exposure to ergot was not ruled out in these instances (Schmidt and Osborn, 1993; Stuedemann and Seman, 2005). Slump refers to the fact that cattle just “ain’t doing right.” Cattle have an unthrifty appearance with rough hair coats that often have not shed from the winter. The sun may bleach out the hair coats. Because of their high body temperatures, cattle spend more time in the shade or watering holes during the day and less time consuming forage. Beef cows consuming E+ fescue produce approximately 50% less milk for their calves, which results in lower weaning weights (Schmidt and Osborn, 1993). At weaning time, calves raised on E+ fescue pasture may be 30–40kg lighter than similar calves on endophyte-free forage (Schmidt and Osborn, 1993). Other potential clinical signs include nervousness, increased salivation, increased rate of respiration, delayed puberty, and reduced conception rates, possibly arising from adverse effects on the male as well as the female (Schmidt and Osborn, 1993; Looper *et al.*, 2009; Strickland *et al.*, 2011). The reduced conception rates are

thought to occur in cattle during the early embryonic period and are generally not associated with late-term abortions or stillborn calves (Thompson *et al.*, 2001). Although sudden deaths during hot summer months have been reported, negligible mortality is associated with summer slump arising from ingestion of endophytic toxins alone, without concurrent exposure to ergopeptine alkaloids produced by *C. purpurea*.

#### *Fescue foot*

Fescue foot refers to a syndrome seen in cattle during the late fall or winter months, which is reported to occur when dietary ergovaline concentrations exceed 400 ppb (Tor-Agbidye *et al.*, 2001). However, the minimum threshold concentrations of ergovaline necessary for the development of fescue foot are very likely temperature dependent and will vary with geographical region. Peripheral vasoconstriction arising from cold environmental temperatures is additive to the vasoconstrictive properties of ergot alkaloids. However, fescue foot is not as common as summer slump, and determinants other than environmental temperature probably exist that help predict whether fescue foot or summer slump is observed clinically. Differences in concentrations of ergovaline or other vasoconstrictive ergot alkaloids, as well as a host of other factors, probably play a role. Vasoconstriction tends to be more severe in rear legs than in the front legs. The switch of the tail and sometimes the tips of the ears are also affected. Vasoconstriction of the back legs is between the coronary band of the hooves and the fetlock area. Areas proximal to the vasoconstriction may be congested, and areas distal to the vasoconstriction undergo gangrenous necrosis, with hooves potentially being sloughed. Affected cattle have visibly swollen rear legs, with shifting rear leg lameness, muscle tremors, rough hair coats, knuckling of the pastern, arching of the back, and eventually, if severe enough, the inability to stand and ambulate (Spiers *et al.*, 2005a,b).

#### *Lipomatosis*

Lipomatosis is a syndrome of fat necrosis affecting abdominal fat stores in mature cattle (Burrows and Tyrl, 2001; Schmidt and Osborn, 1993). If mesenteric fat surrounding intestines is involved, scanty feces, bloat, or intestinal obstruction may result. Perirenal fat may also be affected with or without causing significant clinical problems. Fat in the pelvic canal may become necrotic and hardened and cause dystocia. Although the hardened fat may sometimes be detected by rectal palpation, the discovery is often made at necropsy as an additional finding unrelated to the animal's death. The incidence of fat necrosis in cowherds is highest in those herds with the lowest concentrations of serum cholesterol (Stuedemann and Seman, 2005).

#### *Small ruminants*

Sheep may be affected by fescue and have a syndrome very similar to summer slump in cattle. Ewes grazing fescue have decreased milk production and increased early embryonic mortality (Schmidt and Osborn, 1993; Thompson *et al.*, 2001). Weight gain and skin temperature in young lambs may be decreased (Gadberry *et al.*, 2003). Fescue foot problems are possible in sheep with threshold dietary concentrations of 500 ppb ergovaline at environmental temperatures equal to 7.8°C (Tor-Agbidye *et al.*, 2001). In addition, ergot alkaloids have also been associated with tongue necrosis, along with infertility problems, in sheep (Thompson *et al.*, 2001). There are also reports of goats and deer experiencing lipomatosis (Evans *et al.*, 2004b; Smith *et al.*, 2004).

#### *Horses*

##### *Gestational abnormalities*

Reproductive problems are the most common E+ fescue-related abnormalities noted in horses (Cross, 1997; Blodgett, 2001; Evans, 2011). Late-term abortion is possible but not common. Pregnant mares are most susceptible to adverse effects associated with ingestion of E+ fescue after day 300 of gestation ("average" gestational length of 335–345 days). Lack of prolactin in the late-gestational mare, along with decreased progestagens and higher or lower than normal estrogen concentrations, can cause problems in the mare and/or foal (Cross, 1997; Blodgett, 2001; Evans *et al.*, 2004b; Evans, 2011). Failure to remove mares from fescue pasture or hay during the last month of gestation (30 days prior to a mare's expected foaling date; approximately day 300 of pregnancy) might result in foal abnormalities, prolongation of pregnancy by 20–27 days or more, and/or, at least, agalactia (Cross, 1997; Blodgett, 2001; Evans *et al.*, 2004b). "Fescue foals" can be smaller than average or normally sized and are predisposed to dysmaturity (birth of dysmature or "dummy" foals). Especially in instances of endophytic toxin-induced prolonged gestation, foals can continue to grow and are likely to become "overmature." Overmature foals are similar to dummy foals (i.e., slow to stand and suckle, and predisposed to failure of passive transfer and septicemia), but they are generally larger than "normal"-sized foals, with prematurely erupted teeth and overgrown hooves. In situations involving larger than normal foals, which are often "not ready" for birth and postnatal survival, the mare frequently does not prepare well for parturition, and the incidence of dystocia increases dramatically, resulting in possible uterine, cervical, and/or vaginal trauma. In addition, the chorioallantois (i.e., the portion of the equine fetal membranes diffusely attaching to the uterus) might be thickened and edematous and not rupture at the cervical

star, as is normal. The chorioallantois might also detach prematurely and precede the foal through the birth canal, presenting as a “red bag.” If the foal is able to successfully break out of the edematous chorioallantois and/or amnion and not suffocate, it invariably faces an agalactic mare with minimal colostral antibodies for passive transfer (Cross, 1997; Blodgett, 2001). In these unfortunate circumstances, the mare and/or particularly the foal can die, and if the mare survives, she frequently experiences rebreeding problems. Retained fetal membranes are also more common with fescue toxicosis.

#### *Subfertility*

One study noted increased early equine embryonic mortality with exposure to E+ fescue (Brendemuehl *et al.*, 1994), and depressed endogenous catecholamine activity has been observed in mares exposed to E+ fescue early in gestation (Youngblood *et al.*, 2004). However, other studies have found no increased equine pregnancy loss up to day 300, despite lower progestagen concentrations between gestational days 90 and 120 (Brendemuehl *et al.*, 1996). In general, equine pregnancy rates are usually fairly good on E+ fescue pasture, as long as mares have progressed through the transitional phase in the early spring and are already cycling normally and they are not currently being exposed to ergotized grains or grasses (Evans *et al.*, 2004b). The adverse effects of E+ fescue toxins on stallion reproductive function remain to be demonstrated, although any observed hyperthermia has the potential to decrease semen quality.

#### *Laminitis*

Endophytic toxins can predispose horses to laminitis or painful inflammation of the dermal laminae within the hoof, a condition that can result in potentially life-threatening lameness (Rohrbach *et al.*, 1995).

#### *Camelids (llamas and alpacas)*

Little research has been done with respect to fescue toxicosis in camelid species. Llamas and alpacas can have hyperthermia problems during summer months. Because endophytic toxins can directly affect the thermoregulatory center in the hypothalamus and decrease dermal heat loss via vasoconstriction, E+ fescue should be considered a potential contributor to the heat “stroke” or stress syndrome sometimes seen in llamas and alpacas.

#### *Laboratory rodents*

Although rodents are most likely not naturally exposed to ergopeptine alkaloids, research performed with these species has facilitated initial research evaluating the genomic effects of these compounds (Settivari *et al.*, 2006). Like cattle, rats ingesting E+ rations

experience diminished dry matter intake, making them an appropriate and practical model to evaluate the physiological effects of endophytic toxins associated with summer slump, especially those related to restricted caloric intake (Spiers *et al.*, 2005a,b).

## TREATMENT

### **Nonspecific treatment/prevention for bovine fescue toxicosis**

Cattle experiencing summer slump toxicosis are generally unthrifty and are treated by removal from E+ fescue or by dilution of the ergovaline content of their overall diet. Dilution can be accomplished by feeding supplemental nonfescue forage or concentrates. Clovers or bermudagrass are often oversown into fescue pastures to aid in dilution for a few years (Thompson *et al.*, 2001). Ergot alkaloid concentrations decrease fairly rapidly in E+ fescue following cutting so that waiting at least 1 month after clipping E+ fescue to feed it to cattle is a practical way to control ergovaline exposure (Roberts *et al.*, 2009). Abnormal copper homeostasis related to decreased feed intake can be addressed by copper supplementation (Stewart *et al.*, 2010). Providing shade and water holes can help to cool hyperthermic animals. Some researchers have investigated the use of heat stress-resistant breeds or lineages of cattle to lessen the impact of summer slump. Gangrenous problems with fescue foot are treated with broad-spectrum antibiotics to diminish secondary infection, and the provision of windbreaks or shelter, in particularly cold climates, might be beneficial.

### **Specific treatment for fescue toxicosis in various species**

A D<sub>2</sub> dopamine antagonist that does not cross the blood-brain barrier and cause extrapyramidal side effects, as opposed to perphenazine and metoclopramide, has been developed to treat agalactia and prolonged gestation problems in mares (Cross, 1997). The generic name of the drug is domperidone, and it is marketed as an oral gel called Equidone. It is commonly given orally at 1.1 mg/kg once a day during the week before anticipated parturition if the mare shows no signs of milk production or at a point in time when it is determined that gestation is prolonged. The total dose or dosing regimen can be adjusted if the mare begins dripping colostrum. Agalactia in a mare that has already foaled can be treated once or twice a day with the same dosage mentioned previously until milk flow resumes. Experimentally, cattle



have been treated successfully with domperidone to relieve some of the adverse effects of E+ fescue on cattle production, and other forms of pharmacologic intervention have also been investigated (Jones *et al.*, 2003; Strickland *et al.*, 2011). Domperidone has also been given empirically to agalactic camelids at twice the equine dosage, but there are no peer-reviewed reports of the absorption of domperidone in these species or the efficacy of this treatment approach for agalactia. The Rauwolfian alkaloid reserpine (dose of 2.5–5.0 mg/450 kg horse, once a day), which depletes brain depots of dopamine, serotonin, and/or norepinephrine and causes mild sedation, can be used for the treatment of postparturient agalactia but not for less than anticipated mammary development prior to foaling or prolonged gestation associated with exposures to E+ fescue or other ergopeptine alkaloids (Evans *et al.*, 1999; Evans, 2011).

## PREVENTION

### Prevention of equine fescue toxicosis

#### *Breeding and foaling management*

Knowledge of breeding dates, confirmation of pregnancy, and monitoring of mammary gland development are critical steps in the prevention of equine fescue toxicosis. Removal of the mare from E+ fescue pasture and hay beginning on gestational day 300, during at least 30 days prior to the anticipated foaling date, will also generally prevent agalactia, prolonged gestation, and foaling problems associated with endophytic toxin exposure. Careful evaluation of mammary gland development, supervision of the mare during foaling (with assistance provided as necessary), and observation of the foal for normal postnatal behavior benchmarks, such as the ability to stand and suckle, as well as successful passive transfer, are also essential steps in ensuring that both the mare and the foal have not been exposed to or adversely affected by exposure to tall fescue endophytic ergopeptine alkaloids (Evans, 2011).

#### *D<sub>2</sub> dopamine receptor antagonists*

In some specific instances and geographical location, it is almost impossible to completely remove mares from any exposure to E+ fescue and/or hay. Domperidone can be given once a day to mares kept on fescue during the final 10–14 days before expected parturition (Cross, 1997). The dosage is the same as that for the treatment outlined previously. The D<sub>2</sub> dopamine receptor antagonist fluphenazine (25 mg administered intramuscularly in pony mares on day 320 of gestation) has

also been used to prevent decreases in relaxin related to ergopeptine alkaloid-induced placental dysfunction (Evans, 2011).

### Novel or non-endophyte-infected fescue

Endophyte-infected tall fescue pastures can be replanted with other grasses, such as non-endophyte fescue or new novel varieties of endophyte-infected fescue. Renovation requires a nonselective herbicide (e.g., paraquat and glyphosate) to kill off the pasture grasses and planting with a smother crop for a season before spraying again with a herbicide and replanting with another pasture grass (Roberts and Andrae, 2004). Estimated costs of renovation are approximately \$450/ha (Fribourg and Waller, 2005). Non-endophyte-infected fescue has been used to replace E+ fescue, but it is not very drought, insect, nematode, or herbivore resistant; therefore, stand persistence is not good. Novel endophyte varieties have been developed by infecting endophyte-free varieties of fescue with endophytic strains that produce the peramine of tall fescue but minimal or no ergot alkaloids (Roberts and Andrae, 2004; Fribourg and Waller, 2005). Several varieties have shown promise, including MaxQ and ARK Plus, with the novel varieties having insect and drought resistance without adversely affecting cattle, horses, or sheep. However, caution is warranted until more information is available, given the incidence of equine fescue edema syndrome in Australia. This newly reported syndrome occurred, following drought-like conditions, in horses grazing the Mediterranean variety of tall fescue infected with a novel endophyte (Bourke *et al.*, 2009).

### Ammoniation of hay

Ammoniation of hay will degrade the ergovaline content of hay and make it safe for livestock consumption (Thompson *et al.*, 2001). Ammoniation requires enclosing the hay in an airtight tent structure and pumping anhydrous ammonia gas into the tent for a period of time. Although extremely effective, this process is considered by some to be too time- and labor-intensive, as well as too costly, for routine use. However, there are regions of the United States, where cattle populations are large enough and E+ fescue pastures sufficiently extensive, which might warrant the investment of the labor, time, and money necessary for proper ammoniation E+ fescue hay (Evans *et al.*, 2004b; Roberts and Andrae, 2004).

### Feed supplements

Supplementing livestock with concentrates will decrease the overall dose of ergovaline from fescue. Several different

types of feed additives have also been investigated as ways to ameliorate fescue toxicosis. A glucomannan product (FEB-200) from yeast cell walls that is purported to bind ergovaline in the gastrointestinal tract and prevent its absorption improved cattle performance (Fribourg and Waller, 2005). Another feed supplement from seaweed, Tasco, has been reported to ameliorate some of the oxidative effects and immunosuppression associated with exposure of cattle to E+ fescue (Fike *et al.*, 2001; Saker *et al.*, 2001). Copper supplementation can be beneficial in cattle ingesting E+ fescue (Evans *et al.*, 2004b).

### Pasture considerations

Pastures might be made less toxic by dilution with other grasses or legumes such as bermudagrass or clover, cutting seed heads from the pasture during the most toxic time period of the summer, or increasing the grazing pressure during the summer to avoid seed heads (Stuedemann and Seman, 2005). Provision of plenty of shade, potable water to drink, and water holes in which to cool off or moving cattle to a nonfescue pasture during the warmest summer months are also reasonable management practices that take into consideration pasture circumstances.

## CONCLUDING REMARKS

Tall fescue toxicosis is the major grass-associated forage problem in the United States and is worldwide in its occurrence (Oliver, 1997). Because the toxic principles are actually mycotoxins (i.e., secondary metabolites of a fungus), fescue toxicosis is, in fact, a mycotoxicosis rather than a plant intoxication. The production of the ergopeptine alkaloid ergovaline, potentially the primary toxin responsible for clinical disease, by the endophyte *N. coenophialum* varies seasonally and from year to year. During some years, the ergovaline concentrations in a particular fescue pasture are very high, or there is an increased prevalence of ergotized grasses, including tall fescue. In these circumstances, livestock species, especially cattle and horses, can experience debilitating disease, which can be exacerbated by extremes in ambient temperature. During other years, lower ergovaline concentrations, combined with the nutrient value and hardiness of the endophyte-infected fescue, as well as milder climactic conditions, make its use as a pasture grass or source of hay very practical and, ultimately, extremely profitable. The major disease syndromes associated with fescue toxicosis arise from the prolactin-inhibiting and vasoconstrictive properties of endophytic ergot alkaloids.

Researchers are currently addressing fescue toxicosis in several different ways. One approach is to replace tall fescue with a novel endophyte-infected fescue that is not toxic for animals but is still drought and insect resistant. This has been accomplished, but the costs of pasture renovation are considerable, with some hilly terrain not suitable for renovation. There is also the potential for other, unanticipated toxicoses to be associated with the novel endophyte, under certain growth conditions (Bourke *et al.*, 2009). The other approach to fescue toxicosis has been to find an antidote or preventative for the fescue problem. Domperidone is a suitable treatment or preventative for pregnant mares, but residue concerns will likely prevent its use in the near future for food-producing animals. Other possible preventatives for food animals are still being investigated and include adsorbents, feed supplements, and even vaccines. In addition, newer analytical procedures are being developed, which might help elucidate the precise role of ergovaline, other ergot alkaloids, and their metabolites in the pathogenesis of fescue toxicosis in various livestock species (Strickland *et al.*, 2011).

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# Aflatoxins

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## INTRODUCTION

Aflatoxins (AFs) are considered the most important mycotoxins in human foods and animal feedstuffs on a worldwide scale (Diener *et al.*, 1987; Williams, *et al.*, 2004; Strosnider *et al.*, 2006). Aflatoxin-linked adverse health effects occur in companion animals, livestock, poultry, aquatic animals, humans and other species because aflatoxins are potent hepatotoxins, immunosuppressants, teratogens, mutagens, carcinogens, and are linked with stunted growth and reproductive dysfunctions (Clegg and Bryson, 1962; Allcroft and Lewis, 1963; Robens and Richard, 1992; Eaton and Gallagher, 1994; Bennett and Klich, 2003; Shuaib *et al.*, 2010a). Aflatoxins are the most potent naturally occurring hepatic carcinogens. Many authors are of the opinion that aflatoxicosis in domestic animals is considerably under-identified, and the short- and long-term effects on human health are underestimated. It was estimated by Williams *et al.* in 2004 that ~4.5 billion people have uncontrolled chronic exposure to aflatoxins, and other authors have increased the current estimate to >5 billion people (Khangwiset and Wu, 2010). Due to the potency of aflatoxins there are increasing concerns regarding animal and human exposures. Contamination causes economic losses of corn, cottonseed, peanuts, sorghum, wheat, rice, nuts and spices condiments and other commodities. Losses also occur when processed foods and finished feeds are considered unfit for human and animal consumption.

Worldwide, aflatoxins, because of their prevalence and toxicity, are important in public health with concerns

centering on both primary poisoning from aflatoxins in commodities, food and feedstuffs and relay poisoning from aflatoxins in milk and other animal products. Aflatoxicosis in the human population, especially in areas stricken by poverty, drought and other adverse growing conditions, is an important public health problem and may be increased by climate change (Williams *et al.*, 2004; Vineis and Xun, 2009; Wild and Gong, 2010). Another public health concern is that plant-source illicit drugs may contain aflatoxins, and the interactions between aflatoxins and infectious diseases can contribute to spread of disease in this population.

Many governmental jurisdictions regulate the levels of aflatoxins allowed in animal feedstuffs and human foods because of their toxicity. The allowable levels of aflatoxins in animal feedstuffs and human foods vary with governmental jurisdictions and the perceived risk. For example, the regulated level for aflatoxins in feedstuffs for dairy animals is lower than aflatoxins in feedstuffs fed to beef cattle. Commodities considered unsafe for human foodstuffs are often incorporated into animal feedstuffs (Coppock and Swanson, 1986), and most governments have regulations allowing this diversion. Grain and other seeds used for feeding wildlife can be high in aflatoxins (Henke *et al.*, 2001) and these feedstuffs are generally under-regulated. Regulatory information for aflatoxins is generally posted on government and WHO websites. Regulatory limits for aflatoxins and other mycotoxins have been summarized in the 2003 CAST publication (CAST, 2003).

BACKGROUND

History of aflatoxins

In the late 1950s and early 1960s, aflatoxins were identified as the cause of the mysterious turkey “X” disease in Great Britain (Clegg and Bryson, 1962; Allcroft and Lewis, 1963; Richard, 2008). In the U.S., studies incriminated AFs as the cause of epizootic hepatitis in dogs and as the cause of moldy corn poisoning in pigs (Newberne *et al.*, 1966a,b). Aflatoxins were identified as a potent carcinogen in rainbow trout (Newberne and Butler, 1969). Aflatoxin linkages to human liver disease were made in the 1960s (Robens and Richard, 1992). Since these early discoveries, intensive research has shown and continues to show that the risk of exposure to aflatoxins is a world-wide animal and human health issue. Many authors are of the opinion that other fungal metabolites produced by aflatoxigenic fungi contribute to the toxicology observed under field exposure conditions (Coppock and Jacobsen, 2009).

Aflatoxigenic fungi

Varga *et al.* (2009) have reviewed aflatoxigenic species. Three sections likely account for all the aflatoxigenic species of *Aspergillus*, namely *Flavi*, *Ochraceorosei* and *Nidulantes* (Table 88.1). The most commonly recognized aflatoxigenic fungi are *Aspergillus flavus*, *A. parasiticus* and *A. nomius*; other species of *Aspergillus* have been reported to produce aflatoxins and are given in Table 88.1. Aflatoxigenic species of *Aspergillus* commonly grow in plant-source debris on the soil and can also grow in feedlot manure (Hendrickson and Grant, 1971). Insects

and wind currents spread the spores of aflatoxigenic fungi to plants and these fungi commonly colonize areas of insect damage. Management of insect pests in the order Lepidoptera is a key to reducing aflatoxin contamination in corn, cotton and some tree nuts. In corn infection can take place via silks and the subterranean flowers of peanuts can also be infected without insect injury (Griffin and Garren, 1974; Jones *et al.*, 1980). In addition to plant residues, spores, mycelia, or sclerotia of aflatoxigenic fungi are commonly found in soil, storage areas, processing facilities and in the distribution systems for manufactured products. The production of aflatoxins is associated with spore production by species of *Aspergillus* (Calvo *et al.*, 2002). Strains of *A. flavus* can vary in aflatoxin capability from nontoxic to highly toxigenic and are more likely to produce more aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) than aflatoxin AFG<sub>1</sub>. *A. flavus* and other species can also produce cyclopiazonic acid (CPA). Strains of *A. parasiticus* generally have less variation in toxigenicity and generally produce AFB<sub>1</sub> and varying amounts of AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>. The aflatoxin profile produced by *A. nomius* is considered to be similar to *A. parasiticus*, and like *A. parasiticus*, *A. nomius* is not known to produce cyclopiazonic acid. Recent studies have shown that *A. nomius* is more important as a product of aflatoxins than previously suspected (Johnsson *et al.*, 2008). Aflatoxigenic strains of *Aspergillus* can also produce sterigmatocystin (ST) (Table 88.1).

Conditions for aflatoxin production

The fungi associated with AF production are *A. flavus*, *A. parasiticus* and *A. nomius*, and these fungi are common in most soils and are usually involved in decay

TABLE 88.1 Species of *Aspergillus* identified as producers of aflatoxins (adapted with permission from Varga *et al.*, 2009)

Species <sup>a</sup>	Country	Mycotoxins identified <sup>b</sup>
<i>A. bombycis</i> (F)	Japan, Indonesia	AFB, AFG, KA, A-acid
<i>A. flavus</i> (F)	Ubiquitous	AFB <sub>1</sub> , AFB <sub>2</sub> , KA, CPA, others
<i>A. nomius</i> (F)	U.S., Thailand, S. America	AFB, AFG, KA, others
<i>A. parasiticus</i> (F)	Likely ubiquitous	AFB, AFG, KA, others
<i>A. parvisclerotigenus</i> (F)	Africa	AFB, AFG, CPA, KA
<i>A. minisclerotigenes</i> (F)	U.S., Africa, Australia, S. America	AFB, AFG, KA, CPA, other
<i>A. arachidicola</i> (F)	S. America	AFB, AFG, KA, CPA, others
<i>A. pseudotamarii</i> (F)	Japan, S. America	AFB <sub>1</sub> , KA CPA
<i>A. ochraceoroseus</i> (O)	Africa	AFB <sub>1</sub> , AFB <sub>2</sub> , ST, others
<i>A. rambellii</i> (O)	Africa	AFB <sub>1</sub> , AFB <sub>2</sub> , ST, others
<i>E. stellata</i> (N)	S. America	AFB <sub>1</sub> , ST, others
<i>E. olivicola</i> (N)	S. Europe	AFB <sub>1</sub> , ST, others
<i>E. venezuelensis</i> (N)	S. America	AFB <sub>1</sub> , ST, others

<sup>a</sup>Section and F = Flavi, O = Ochraceorosei and N = Nidulantes.  
<sup>b</sup>AFB = aflatoxin B, AFG = aflatoxin G, KA = kojic acid, CPA = cyclopiazonic acid, ST = sterigmatocystin. For others see Varga *et al.* (2009).

of plant materials (Moss, 1991; Smith and Ross, 1991; Jacobsen *et al.*, 2007). The general growth conditions for aflatoxigenic species are described as requiring moisture contents in equilibrium with 80–85% or more and temperatures of 13 to 42°C with optimum growth at 25° to 37°C (Jacobsen *et al.*, 2007). For example, the critical moisture content for growth of *A. flavus* in starchy cereal grains is 17 to 18%, soybeans 17 to 17.5% and for peanuts is 9 to 10.5% (Sauer *et al.*, 1992). The upper limit of moisture for growth of *A. flavus* and AF production is about 30%. *Aspergillus flavus* will grow slowly below 13°C, and most rapidly at 37°C, but does not produce AFs at temperatures below 13°C or above 42°C. In addition to temperature and equilibrium relative humidity, other factors that influence aflatoxin production are carbon, nitrogen, plant metabolites and sugars in substrates. High maximum and high minimum temperatures with high net evaporation are more important triggers than temperature and humidity alone. High aflatoxin production generally occurs when the fungus grows in the seed embryo. Both epigenetic and genetic factors affect aflatoxin production and the interactions are not well understood (Bhatnagar *et al.*, 2006). Aflatoxigenic *A. flavus* isolates vary from 40 to >70% of the total *A. flavus* population (Campos *et al.*, 2008). This observation has led to the use of atoxigenic *A. flavus* strains to preemptively colonize infection sites such that toxigenic strains are prevented from infecting and growing (Atehnkeng *et al.*, 2008). The percent of aflatoxigenic isolates can vary between ingredients and finished feeds, and finished feeds that have spoiled can have high primary production of aflatoxins. Growth of aflatoxigenic fungi and aflatoxin production can rapidly occur especially in damaged high moisture seeds. Experimental study on maize with 57% damaged kernels had 25 ppm AFB<sub>1</sub> at 6.5 day (Seitz *et al.*, 1982). High levels of aflatoxin production can occur in maize before a 0.5% loss in dry matter occurs. High levels of aflatoxins in corn are not linked to the production of kojic acid (black light fluorescence).

### Moisture management of grain

The most common methods of preventing the growth of aflatoxigenic fungi in dry-stored commodities are by controlling the moisture level and temperature. Water available for aflatoxigenic fungi in stored grain is best indicated by equilibrium relative humidity or water activity ( $a_w$ ) in the air around the grain (Caddick, 2003). The  $a_w$  is equal to percent relative humidity/100. Temperature of the grain is important as the  $a_w$  increases with temperature. In cereal grains aflatoxigenic fungi generally cannot grow when the  $a_w$  is below 0.80. A lower  $a_w$  is required for protection of oilseeds. Oilseeds have more available water on the seed surface

because the seed absorbs less moisture. Increasing the oil fraction of the seed decreases the water that is absorbed into the seed. For most cereal grains, storage moisture of 12% is recommended for a wide range of grain temperatures, and for oilseeds the storage moisture from <8 to 9% is recommended for a wide range of temperatures inside the storage unit since other species of *Aspergillus* can grow in commodities at lower  $a_w$  than *A. flavus*. These fungi produce both heat and metabolic water as they grow and this can create a favorable moisture and temperature for *A. flavus* to grow (Sauer *et al.*, 1992).

Measuring moisture with a grain tester provides the average moisture of the kernels. This measurement can misinterpret the storability of grain. If the moisture is heterogeneously distributed among the seeds or pellets, hot spots can occur. This phenomenon occurs because a microbial microcosm exists around each high moisture seed and that microcosm can meet the requirements for fungal growth and mycotoxin production. This is the reason why high and low moisture grain should not be blended. In the microcosm with a favorable  $a_w$  level, growth of aflatoxigenic fungi can occur. The metabolism of microorganisms in the microcosms produce moisture and some microorganisms can also produce heat. Insects and other pests in grain also produce moisture and hot spots can form because of insect and other pests in stored grain. Monitoring multiple areas of a storage unit is essential for preventing the production of mycotoxins. Samples for aflatoxin analyses should be placed in moisture-proof bags to prevent desiccation. As soon as possible after sampling, the individual moisture content of at least 100 seeds or pellets should be determined to identify moisture distribution in the kernels or pellets. This test result provides valuable insights into the storability of the commodity.

### In vivo production of aflatoxins

*Aspergillus flavus* and *A. fumigatus* have also been identified as pathogens in animals and humans (Barton *et al.*, 1992; Drakos *et al.*, 1993; Pepelnjak *et al.*, 2004). Aflatoxins can be produced in tissues by toxigenic fungi. Assays of cultured *A. flavus* and *A. fumigatus* isolated from tissues have shown these fungi can produce AFs, and chemical analyses of infected tissues have shown aflatoxins to be present (Matsumura and Mori, 1998; Mori *et al.*, 1998; Pepelnjak *et al.*, 2004). Aflatoxins being produced in tissues have not been shown to cause liver lesions typical of aflatoxicosis. Typically, aflatoxigenic fungi infect animals and humans that are immunocompromised. Systemic aspergillosis by aflatoxigenic fungi was considered to contribute to immunosuppression (Mori *et al.*, 1998). *A. nomius* has been identified as an etiology of keratitis.

## PROVENANCE OF AFLATOXINS IN FEEDSTUFFS AND FOODS

### Sources and occurrences of aflatoxicosis

Almost any food or feedstuffs that can support the growth of aflatoxigenic fungi can contain aflatoxins. Aflatoxins and their metabolites can be relayed to edible animal products. Occurrences of contaminated cows', goats' and human milk (AFM<sub>1</sub>) for human consumption have been identified. Essentially all species of domestic animals have been poisoned with aflatoxins under field conditions. Outbreaks of aflatoxicosis in pet foods including birdseed have occurred and may be under-reported (Leung *et al.*, 2006; Boermans and Leung, 2007; Rumbelha and Morrison, 2010). Birdseed and other feedstuffs for wildlife have been reported to be high in aflatoxins (Thompson and Henke, 2000; Henke *et al.*, 2001). The sources of the aflatoxins in pet foods have been primarily maize and maize byproducts, rice, millet and peanut byproducts. Food garbage, moldy bread and other diverted foods can be a source of AFs. Tree nuts, peanuts, figs, oilseeds, tobacco, coconut, condiments and spices, cereal grains, eggs, cheese and many other commodities, feedstuffs and foodstuffs have been shown to contain aflatoxins.

All cereal crops and oilseeds can contain aflatoxins and the determinant is the growth of aflatoxigenic fungi. These fungi prefer minimum temperatures of >25°C, with the abiotic and biotic microclimate of a geographic region having major effects on the genera and species of aflatoxigenic *Aspergillus* present and on production of aflatoxins (Cotty and Jaime-Garcia, 2007). Aflatoxin contamination of commodities in the field can be divided into two distinct phases. Growth of aflatoxigenic fungi and mycotoxin production occurring preharvest in the developing crop and post-harvest mycotoxin production after the crop has matured and has been harvested. In the preharvest phase, wounds in the developing crop including those caused by insects, birds, mammals and hail provide entry points for aflatoxigenic fungi. The importance of Lepidopteran insect damage is demonstrated by reductions of aflatoxin content in maize and cotton where transgenic (Bt) maize and cotton are grown, or a reduction in peanuts where the lesser stalk borer numbers are reduced, or in almonds or pistachio nuts where insects such as the orange navel worm are controlled (Mahoney and Rodriguez, 1996; Cotty *et al.*, 1997; Schatzki and Ong, 2001; CAST, 2003; Wu, 2006). Excessive heat and drought may decrease the native resistance of plants to aflatoxigenic fungi by physiological and anatomical mechanisms. Insect damage to ears, bolls, pods or nuts has been shown to dramatically increase the risk of aflatoxin production in maize, cotton, peanuts, almonds and pistachios. The post-harvest fungal invasion and aflatoxin production can occur

from maturation in the field to the point of human and animal consumption. Post-harvest aflatoxin contamination can occur when the commodity is suitable for growth of aflatoxigenic fungi at harvest during transport, storage and manufacturing. Aflatoxin contamination in wheat is essentially a post-harvest phenomenon (Jacobsen, 2010) whereas maize contamination can be either preharvest or post-harvest (Payne, 1992). Delayed harvest due to wet conditions with sufficient heat to support growth of aflatoxigenic fungi can result in high levels of aflatoxins being produced. In some tree nuts such as walnut, almond and pistachio and in cotton a natural opening in the nut hull or boll combined with delayed harvest and warm, humid conditions can result in significant aflatoxin contamination. Delayed irrigation and resulting drought stress at silking can increase the risk for aflatoxin production in maize. Damage to kernels or nuts during harvest, cleaning and general grain handling weakens the mature seeds to fungal invasion. The suitability of grain for storage at harvest is adversely affected by moisture content, physical damage to the kernels and the extent to which fungi have invaded the seed before storage (Johansson *et al.*, 2006, 2008; Coppock and Jacobsen, 2009). Moisture level is a risk factor for mycotoxins in stored commodities (Sauer *et al.*, 1992; Frisvad, 1995; Wicklow, 1995; Jacobsen *et al.*, 2007; Dorner, 2008). Blending grain of high moisture levels (moisture considered too high for safe storage) with grain of lower moisture levels (safe for storage) can produce conditions in the grain mass that favor storage mold growth and mycotoxin production (Coppock and Christian, 2007; Coppock and Jacobsen, 2009). The migration of moisture from the high moisture seeds occurs at a slow rate. Moisture migration is dependent on moisture level in the air trapped between the seeds with seeds of lower moisture content taking up the moisture from the seeds with higher moisture content. The high moisture seeds provide a niche with a high water activity ( $a_w$ ) that is favorable for the growth of toxigenic and other fungi, which if allowed to grow, produces additional moisture and heat via microbial metabolic activity. The probability of fungal infection in seeds increases when the moisture content and temperature of the seed is increased. In nuts the acquired inoculum remains with the whole and shelled nut and the low water activity ( $a_w$ ) prevents fungal growth and mycotoxin production (Bayman *et al.*, 2002). Intensive cropping practices and decreased genetic diversity probably contributes to increased preharvest infections of commodities with fungi that produce aflatoxins (Lillehoj, 1992; Brown *et al.*, 1999).

### Rapid formation

Aflatoxin formation can rapidly occur (Coppock *et al.*, 1989). Lee *et al.* (1986) showed that maturing corn kernels



in the ear on the plant inoculated with *A. flavus* had 0.3 to 2 ppb aflatoxins present at 2 days after inoculation, 950 to 2800 ppb aflatoxins present at 4 days after inoculation and 3600 to 4500 ppb aflatoxins present at 7 days after inoculation. The *A. flavus* infection reduced the starch content of the kernel by 12%. Kojic acid was present at 2700 ppb 16 hours (h) after inoculation. High moisture corn and sorghum lightly inoculated with *A. flavus* was shown to produce ppm levels of aflatoxins within 48 h (Winn and Lane, 1978). Over-mature sweet corn was shown to contain 2.36 ppm AFB<sub>1</sub> and 0.21 mg of AFB<sub>2</sub> (Hall *et al.*, 1989). Sweet corn has been considered more hazardous than field corn because of its higher free sugar content.

## Oilseeds

Cottonseed can be a source of aflatoxins in animal diets. Preharvest contamination of cottonseed occurs (Jaimi-Garcia and Cotty, 2003). Insect damage, timing of irrigation or rain, relative humidity around the bolls, stage of maturity and variety of cotton can be factors in causing preharvest contamination of cottonseed with aflatoxins (Russell *et al.*, 1976; Lillehoj *et al.*, 1987). In stored cottonseed growth of aflatoxigenic fungi may occur when the average moisture level in stored cottonseed is greater than 7 to 8%. The lipids and protein in cottonseed enhances AF production (Mellon and Cotty, 1998; Mellon *et al.*, 2000). Peanut hay, peanuts and peanut byproducts are an important source of AFs (McKenzie *et al.*, 1981; Cullen and Newberne, 1994). Aflatoxins generally are the most concentrated in the seeds. The growth of aflatoxigenic fungi can occur in stored peanuts when moisture exceeds 8% and ambient temperature is above 25°C. Drought-stressed peanuts have decreased native resistance to infection by aflatoxigenic fungi (Wotton and Strange, 1987). Phytoalexin produced by the infected peanut seed increased and inhibited the growth of *A. flavus*, but levels of AFs continued to increase for an additional day. Drought-stressed peanut seeds have decreased production of phytoalexin, and aflatoxin production in drought-stressed peanut kernels is limited by available moisture. *A. flavus* can grow on soybeans at  $a_w$  of 0.77 (Trucksess *et al.*, 1988).

## Distiller's grains and commodity byproducts

Distiller's byproducts can be a source of aflatoxin (Hesseltine, 1984; Anom, 2006; Wilkinson and Abbas, 2008). Using aflatoxin-contaminated commodities for fuel alcohol production can salvage corn and other high starch commodities. Fermentation does not destroy AFs. On a dry matter basis, the concentration of AFs in the stillage, compared to AFs in the feedstock, is increased three- to four-fold due to the loss of starch.

Approximately 40% of the AFs are in the syrup (distiller's solubles) fraction and 60% are in the solids fraction. Aflatoxins generally are not found in the alcohol fraction. Depending on the processes, commodity byproducts can have AF levels that exceed the levels in the raw commodity (Bullerman and Bianchini, 2007).

## Chemistry

Aflatoxins have a difuranocoumarin chemical structure (Figure 88.1). Approximately 18 aflatoxins have been chemically characterized. Aflatoxins are in two chemical groups, the difurocoumarocyclopentenone series (includes AFB<sub>1</sub>, AFB<sub>2</sub>, AFB<sub>2A</sub>, AFM<sub>1</sub>, AFM<sub>2</sub>, AFM<sub>2A</sub> and aflatoxicol) and the difurocoumarolactone series (includes AFG<sub>1</sub> and AFG<sub>2</sub>). The "B" Group fluoresce blue in long wavelength ultraviolet light and the "G" Group fluoresce green. The primary aflatoxins of concern in feedstuffs are AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>. Analytical results for aflatoxins generally are the sum of the concentrations of these four toxins. AFB<sub>1</sub> is the most potent aflatoxin and this chemical form is generally the most abundant in feedstuffs and foods. The order of toxicity is AFB<sub>1</sub> > AFG<sub>1</sub> > AFB<sub>2</sub> > AFG<sub>2</sub>. Hydroxylated aflatoxin metabolites are excreted in milk and the important metabolites are AFM<sub>1</sub> and AFM<sub>2</sub> (Garrido *et al.*, 2003; Wu *et al.*, 2009). AFM<sub>1</sub> is the toxic metabolite of AFB<sub>1</sub> and AFM<sub>2</sub> is the hydroxylated form of AFB<sub>2</sub>. Although AFM<sub>1</sub> and AFM<sub>2</sub> are commonly associated with milk and other edible animal products, aflatoxigenic fungi can also produce these compounds. The regulated level of AFs generally is the total mass of AFB<sub>1</sub> + AFG<sub>1</sub> + AFB<sub>2</sub> + AFG<sub>2</sub>. Versicolorin A and sterigmatocystin mycotoxins are intermediates in the synthesis of aflatoxins. Cyclopiazonic acid is produced by aflatoxigenic strains of *A. flavus*. Other

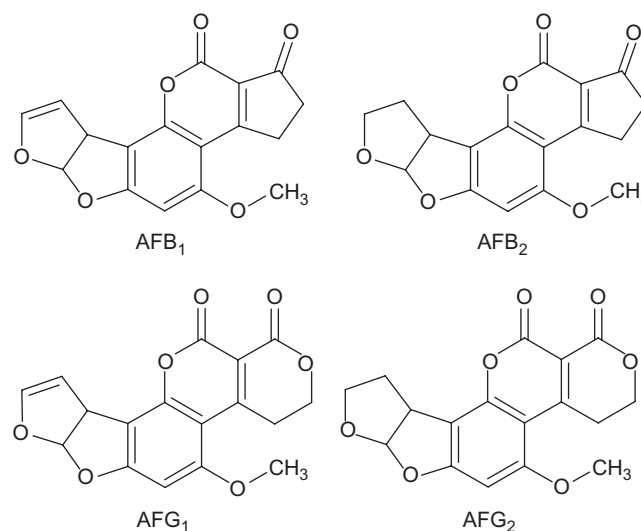


FIGURE 88.1 Chemical structures of aflatoxins.

mycotoxins can be produced by aflatoxigenic strains of *Aspergillus* (Table 88.1) (Varga *et al.*, 2009).

## SAMPLING AND ANALYSIS

The majority of the variation observed in taking multiple samples from a shipping vessel or storage unit occurs with sampling (Whitaker, 2003). Aflatoxins in the bulk lot of feedstuffs are generally assumed to be the same as the mycotoxins identified in the sample. For this reason the samples obtained should be representative of the feedstuffs. A representative sample may be difficult to obtain because the distribution of aflatoxins in the suspect feedstuff or foodstuff can be highly heterogeneous. For this reason, a single random sample may not represent the aflatoxin levels in the feedstuffs sampled. A small percentage of the kernels in a lot of wheat, barley, corn, cottonseed, peanuts and other feedstuffs and foodstuffs may be contaminated with very high concentrations of aflatoxins. Sampling error can occur even with a well-designed sampling protocol. Interpretations of analytical findings should reflect the sampling procedure, and aflatoxicosis cannot be ruled out based on negative chemical analyses of feedstuffs. Another sampling problem is that the available feedstuff for laboratory testing may be limited because livestock or poultry has consumed the suspect feed. In these situations feed may be available from the edges of the feed trough, or clumps of feed retained in the feed handling equipment. Aflatoxin can be present in clumps of feedstuffs (Crockcroft, 1995). These clumps may contain high levels of aflatoxins and only a few animals may have consumed feed from this source, or the clumps were mixed with the feed and fed to a large number of animals. The toxicologist should know the sampling and analytical method(s) used and the limitations of these methods before interpreting analytical results. For the analytical results it is important to know the method(s) employed, the reference standards used including internal standards and an estimate of the percent extracted from the matrix. If this information is not available or is not known to the toxicologist, a risk exists in misinterpreting the qualitative and quantitative results.

## PHARMACOKINETICS/ TOXICOKINETICS

### Absorption

Aflatoxins are efficiently absorbed by passive diffusion from the gastrointestinal tract and are primarily

transferred from the intestine to the hepatic portal blood (Hsieh and Wong, 1994). Very few aflatoxins appear to be transferred into the lymphatic system (Kumagai, 1989). Young animals absorb AFs more efficiently than older animals. Rats at 2.5 weeks of age absorbed AFB<sub>1</sub> at a rate ~15 times greater than rats 4 to 5 weeks of age. Cattle were given a single oral dose of AFs from rice culture (42% AFB<sub>1</sub> and 27% AFB<sub>2</sub>) in gelatin capsules (Cook *et al.*, 1986). AFB<sub>1</sub> and AFM<sub>1</sub> were observed in venous blood (jugular vein) 30 minutes after dosing and reached maximal levels 4 to 8 hours after dosing. The maximal levels of AFM<sub>1</sub> occurred before AFB<sub>1</sub> and the maximal blood levels of AFB<sub>1</sub> generally were higher than AFM<sub>1</sub>. These findings suggest that AFs are rapidly absorbed from the rumen. Rats have been shown to absorb AFB<sub>1</sub> most efficiently from the duodenum and jejunum (Kumagai, 1989). The rate of absorption of AFB<sub>1</sub> in rats is dependent on concentration providing evidence that AFs are absorbed by passive diffusion. The rate of AFB<sub>1</sub> absorption from the duodenum of rats in diestrus was greater than the rate of absorption of AFB<sub>1</sub> from the duodenum in rats in mid-lactation. Aflatoxins can be bound to adsorbents in the gastrointestinal tract.

### Metabolism and excretion

Biotransformation is important in the toxicology of AFs. The most important organ for biotransformation is the liver, and biotransformation can also occur in the kidney and intestinal tract. The majority of research has focused on the biotransformation of AFB<sub>1</sub>. With the exception of the AFB<sub>1</sub>-8,9-epoxide, the biotransformation products are less toxic than AFB<sub>1</sub>. Cytochrome P450 (AFB<sub>1</sub> hydroxylase) has a key role in the biotransformation of AFB<sub>1</sub> to AFB<sub>1</sub>-8,9-epoxide. The formation of AFB<sub>1</sub>-8,9-epoxide is considered to be the most significant biotransformation pathway because the AFB<sub>1</sub>-8,9-epoxide forms adducts with DNA, RNA and proteins. Conjugation of AFB<sub>1</sub>-8,9-epoxide with glutathione (GSH) is considered to be an important detoxification pathway. Other biotransformation products of AFB<sub>1</sub> are AFQ<sub>1</sub> which can be metabolized to AFH<sub>1</sub>. AFB<sub>1</sub> is also metabolized to AFP<sub>1</sub>, AFM<sub>1</sub>, aflatoxicol and other metabolites. The AFP<sub>1</sub>, AFM<sub>1</sub>, AFQ<sub>1</sub> and aflatoxicol form glucuronide and sulfate conjugates.

Turkeys activate AFB<sub>1</sub> ~3.5 times more than chickens by homologs to human CYP1A2, CYP1A5 and other CYPs to form AFB<sub>1</sub>-8,9-epoxide (Yip and Coulombe, 2006; Rawal *et al.*, 2010). Younger birds activate AFB<sub>1</sub> at a greater rate than older birds. Conjugation of the AFB<sub>1</sub>-8,9-epoxide by GSH is considered by some authors to be the rate-limited determinant in species susceptibility to AFB<sub>1</sub> rather than the rate at which AFB<sub>1</sub>-8,9-epoxide is formed. It is likely that there are interactions

between the rate at which AFB<sub>1</sub>-8,9-epoxide is formed and detoxication by GSH. The affinity of the glutathione S-transferases for AFB<sub>1</sub>-8,9-epoxide is also important.

Cattle were given single oral doses (ranging from 0.2mg, 0.4mg, 0.6mg and 0.8mg/kg body weight) of AFs from rice culture (42% AFB<sub>1</sub> and 27% AFB<sub>2</sub>) in gelatin capsules (Cook *et al.*, 1986). AFM<sub>1</sub> was identified in rumen contents 2h after dosing. This observation suggests that AFM<sub>1</sub> is formed by the flora and fauna in the rumen. Urine from these cattle contained AFB<sub>1</sub> and AFM<sub>1</sub>. Kuilman *et al.* (1998, 2000) showed that bovine hepatocytes metabolized AFB<sub>1</sub> to AFM<sub>1</sub> (48% metabolized in 8h), AFB<sub>1</sub> dihydrodiol and minor levels of AFB<sub>1</sub> glutathione conjugate. Liver preparations from calves previously treated with  $\beta$ -naphthoflavone had increased *in vitro* transformation of AFB<sub>1</sub> to AFM<sub>1</sub> when compared to liver preparations from calves that had not been previously treated with  $\beta$ -naphthoflavone (Bodine *et al.*, 1982). Hatch *et al.* (1982) showed that hepatic damage by AFB<sub>1</sub> in goats was increased by pretreatment with phenylbutazone and benzoflavones. Hepatic Kupffer and endothelial cells in the rat have been shown to have up-regulated activity of AFB<sub>1</sub> hydroxylase, the enzyme that converts AFB<sub>1</sub> to AFM<sub>1</sub> (Gemechu-Hatewu *et al.*, 1996). The P450 inducers used were phenobarbital, isosafrole, Aroclor 1254 and 3-methylcholanthrene.

Aflatoxins are excreted in milk, eggs, urine, semen, bile and feces. Excretion of AFs in milk is discussed in the following sections (see Residues). Bingham *et al.* (2004) identified AFM<sub>1</sub> and AFP<sub>1</sub> in dog urine. AFB<sub>1</sub>, AFM<sub>1</sub> and aflatoxicol have been shown to be formed by hens and are transferred to the egg (Oliveira *et al.*, 2000, 2003). Dogs dosed with 100 $\mu$ g AFB<sub>1</sub>/kg body weight excreted AFM<sub>1</sub> in the urine and 90% of the AFM<sub>1</sub> excreted occurred within 12h after dosing (Bingham *et al.*, 2004). Humans exposed to dietary aflatoxins excrete ABM<sub>1</sub>, AFP<sub>1</sub> and AFB<sub>1</sub>-N<sup>7</sup>-Guanine in urine (Groopman *et al.*, 1985). Aflatoxins have been shown to be excreted in boar semen (Picha *et al.*, 1986). Rats excrete AFB<sub>1</sub> into the intestinal tract via bile and the intestinal mucosa also excretes aflatoxins (Kumagai, 1989).

## Residues

Residues of AFs, especially AFM<sub>1</sub>, can occur in edible animal products. The foodstuffs of greatest public health concern are milk, dairy products, especially those made from casein, and liver and kidney (Applebaum *et al.*, 1982). Aflatoxins can be present in meat. Most governmental jurisdictions regulate the permissible level of AFs in edible animal products. In addition to relay from feedstuffs to edible animal products, AFs can be formed in animal products that support or have supported the growth of aflatoxigenic fungi.

## Milk

Aflatoxins can be present in processed milk products. Issues regarding aflatoxin contamination of fluid milk generally increase when regional contamination of commodities (e.g., corn, sorghum, cottonseed) occurs before harvest (Applebaum *et al.*, 1982). Milk products such as cheese and dried milk also can become directly contaminated with AFs by fungal growth in these substrates. Aflatoxins appear in cows' milk within 12 to 48h after ingestion (Applebaum *et al.*, 1982; Frobish *et al.*, 1986). The amount of dietary AFs being excreted in milk varies with the high value being reported as 3% and the low value being reported as 0.17% (Applebaum *et al.*, 1982; Stubblefield *et al.*, 1983; Price *et al.*, 1985; Frobish *et al.*, 1986). To compare with other mammalian species, humans excrete 0.09 to 0.43% of dietary AF intake in breast milk, sheep excrete 0.26 to 0.33% of the dietary AFB<sub>1</sub> as AFM<sub>1</sub> in the milk, and AFs in sows' milk have been shown to be ~1000-fold less than the AFs in the diet (Zarba *et al.*, 1992; Silvotti *et al.*, 1997; Battacone *et al.*, 2005). The stage of lactation is important for excretion of aflatoxins (Veldman *et al.*, 1992). The percentage of AFs in milk has been reported to increase with increasing milk yields (Frobish *et al.*, 1986). Cows in early lactation can excrete from 3.8 to 6.2% of the dietary AFB<sub>1</sub> as AFM<sub>1</sub> in the milk, and cows in late lactation can excrete 1.8 to 2.5% of dietary AFB<sub>1</sub> as AFM<sub>1</sub>. This phenomenon is independent of the level of AFB<sub>1</sub> in the diet and appears to be linked to cows having up-regulation of AFB<sub>1</sub> metabolism during early lactation. The phenomenon of excreting more AFM<sub>1</sub> in milk during early lactation must be considered in calculating safe dietary levels of aflatoxins for dairy cattle, and may be of importance in calculating the rations for other milk-producing animals. Herd-specific analytical data can be used to calculate the dietary:milk ratio for AFs. Our experience has shown that this approach is accurate to predict aflatoxins in milk after changes have been made to reduce dietary aflatoxins. The dietary threshold for aflatoxin excretion in cows' milk appears to be ~15ppb dietary level or 230 $\mu$ g AFB<sub>1</sub>/cow/day. Aflatoxins in milk generally disappear within 24 to 72h after all the aflatoxins have been removed from the diet.

Sheep and swine excrete AFs in milk. Residues of AFM<sub>1</sub> in milk have been studied in dairy sheep (Battacone *et al.*, 2003, 2005, 2009). The ratio of AFB<sub>1</sub> in the diet to AFM<sub>1</sub> in milk was not affected by the dose of aflatoxins. Sheep receiving a single dose of AFB<sub>1</sub> excreted 0.26 to 0.33% of the dose as AFM<sub>1</sub> in milk. Aflatoxin level in curds was two times higher than milk. Aflatoxins are excreted in sows' milk (Silvotti *et al.*, 1997). The levels of aflatoxins in sows' milk increased over a 25-day lactation period. The sows were fed diets containing 800ppb AFB<sub>1</sub> or a diet containing 400ppb AFB<sub>1</sub> + 400ppb AFG<sub>1</sub> for the entire gestation and



TABLE 88.2 Levels in ng/g or ng/ml of AFs in tissues after steers were fed a diet containing 800ppb AFs for 17.5 weeks (Richards *et al.*, 1983)

Toxin	Liver	Kidney	Muscle	Heart	Lung	Rumen
AFB <sub>1</sub>	0.37	0.09	0.002	0.004	0.014	13.05
AFM <sub>1</sub>	1.07	4.82	0.115	0.14	0.29	1.66

lactation period. The average ratio of aflatoxins in the diet to aflatoxins in milk was ~1000:1. Sows fed diets containing 800ppb AFB<sub>1</sub> or 400ppb AFB<sub>1</sub> + 400ppb AFG<sub>1</sub> excreted AFB<sub>1</sub> or AFB<sub>1</sub> + AFG<sub>1</sub>, respectively. Sows fed AFB<sub>1</sub> excreted AFM<sub>1</sub> and sows fed AFB<sub>1</sub> + AFG<sub>1</sub> excreted AFM<sub>1</sub> + AFM<sub>2</sub> metabolites.

The general consensus in the scientific literature is that aflatoxins are stable in milk, and are primarily associated with milk protein. Aflatoxins in raw milk slowly decrease by 11 to 25% in 1 to 3 days of storage at 5°C. Approximately 75% of aflatoxins in milk are found in the casein (protein) fraction and 25% in the whey fraction of milk. Aflatoxins are also found in butter and the division is ~84% in butter milk and ~16% in butter. Aflatoxins are concentrated in cheese, and the concentration factor varies with the type of cheese and cheese-making procedures. AFM<sub>1</sub> is decreased during yogurt manufacturing and decreasing the pH of yogurt decreases the levels of AFM<sub>1</sub> (Govaris *et al.*, 2002).

### Residues in meat

Aflatoxins generally are found in liver, kidney and edible parts of the gastrointestinal tract. Aflatoxins are not known to accumulate in the body fat. Two-week-old turkey poults were fed diets containing 50 and 150ppb AFB<sub>1</sub> (Richard *et al.*, 1986). The poults were killed at 11 weeks, 13 weeks and 1 week or 2 weeks after being fed the 50 and 150ppb diets for 11 weeks (1 or 2 weeks on aflatoxin-free diet). At 50ppb feeding level for 11 weeks AFB<sub>1</sub> was found in liver (0.02 to 0.09ng/g), kidney (0.01 to 0.02ng/g) and gizzard (0.043 to 0.162ng/g), and AFM<sub>1</sub> was not shown to be in liver, kidney and gizzard. Feeding 50ppb aflatoxins for 13 weeks increased the residues of AFB<sub>1</sub> and AFM<sub>1</sub>; for AFB<sub>1</sub> liver contained 0.02 to 0.13ng/g, kidney contained 0.01 to 0.34ng/g and gizzard at trace to 0.113ng/g; AFM<sub>1</sub> in liver was 0.11 to 0.14ng/g and kidney contained 0.01 to 0.07ng/g. At the 150ppb feed level, fed for 11 weeks, AFB<sub>1</sub> in liver was 0.08 to 0.13ng/g, kidney was 0.025 to 0.08ng/g and gizzard trace to 0.22ng/g; AFM<sub>1</sub> was 0.03 to 0.10ng/g in liver and 0.09 to 0.13ng/g in kidney. Aflatoxin M<sub>1</sub> was not shown to be present in the gizzard. Breast and thigh muscles did not contain AFs. Birds on the 150ppm diet for 11 weeks and the control diet (no aflatoxins) for 2 weeks essentially had cleared the aflatoxins from the liver, kidney and gizzard. Chickens

fed a diet containing rice culture that had supported growth of *A. parasiticus* only produced AFB<sub>1</sub> (Pandey and Chauhan, 2007). At 2 weeks of age groups of birds were placed on diets containing minimum levels of 0.0ppm, 2.5ppm, 3.13ppm and 3.91ppm AFB<sub>1</sub>, respectively. These diets were fed for 40 weeks. Tissues and eggs were collected on day 40 for aflatoxin analyses. AFB<sub>1</sub> in eggs increased with feed levels, the mean levels were 1.43ppb, 1.39ppb and 1.63ppb, respectively, for the different treatment groups. Levels in breast meat were 18ppb, 26ppb and 26ppb, respectively, for the chickens in the different treatment groups. The levels of aflatoxin metabolites were not reported, and renal and liver histopathology was reported. Young birds have higher levels of AFs and require longer to clear AFs (Hussain *et al.*, 2010). The translocation of AFB<sub>1</sub> from diet to eggs was studied in 12-week-old hens given diets containing 0, 100, 300 or 500ppb AFB<sub>1</sub> (Oliveira *et al.*, 2000). AFB<sub>1</sub> was only detected at levels from 0.05 to 0.16ppb (mean 10ppb) in the eggs from hens on the 500ppb diet. In this study the transfer rate was 5000:1 diet to egg ratio.

The translocation of AFs into edible tissues of pigs has been studied. Jacobson *et al.* (1978) fed feeder pigs (54 to 72kg body mass) diets containing pure AFB<sub>1</sub> at 100, 2000 and 400ppb. The basal diet contained 0.1ppb AFB<sub>1</sub> and 0.1ppb AFB<sub>2</sub>. The diets were fed for 4 weeks. Liver, kidney and skeletal muscle contained levels of AFB<sub>1</sub> that were correlated with the feeding level. In decreasing dietary level, AFB<sub>1</sub> levels in liver were 1.5, ~0.5 and ~0.2ppb; levels in skeletal muscle were ~1, ~0.5 and ~0.2ppm; and levels in kidney were 4.4, ~0.7 and ~0.2ppb.

Steers fed a diet containing 800ppb aflatoxins for 15 weeks and then placed on an aflatoxin-free diet for 2.5 weeks did not have residues of aflatoxin in heart, skeletal muscle, liver and kidney (Richard *et al.*, 1983). Steers fed a diet containing 800ppb aflatoxins for 17.5 weeks did have residues of aflatoxins (Table 88.2).

## TOXICODYNAMICS

### Mechanisms of action

The metabolism of aflatoxins, unlike many other mycotoxins, is closely linked with toxicity (Eaton and Gallagher, 1994). AFB<sub>1</sub> is metabolized in a P450-dependent



reaction to AFB<sub>1</sub>-8,9-epoxide which forms adducts with macromolecules in the cell. The affinity of AFB<sub>1</sub>-8,9-epoxide in decreasing order for macromolecules is DNA > RNA > protein. The formation of these adducts is considered important in the toxicity of aflatoxins. The DNA adduct is formed with N<sup>7</sup>-guanine and this adduct is relatively resistant to DNA repair processes. Up-regulation of the P450 system increases the toxicity of aflatoxins. Species sensitivity to aflatoxins is linked to the rate of bioactivation and the rate of detoxification. AFB<sub>1</sub>-8,9-epoxide is primarily detoxified by Phase II synthetic reactions by GSH. In mammals glutathione S-transferase mediated conjugation is an important detoxification pathway, and species differences in enzyme activity in part account for differences in species susceptibility. Other Phase II pathways that decrease the toxicity of aflatoxins include formation of glucuronide and sulfates.

### Immunotoxic effects

The immunotoxic effects of aflatoxins are of public health and economic importance. The native resistance to disease is reduced and vaccine protection is also impaired. The apparent clinical signs of aflatoxicosis may be limited to increased occurrences of infectious disease. Recovery from infectious disease may be prolonged and require additional treatments. A clue to immune dysfunction is often infections caused by organisms that generally are not considered to be pathogens. Studies have shown that the immunotoxicity of AFs could be a teratogenic effect of prenatal or pre-hatching exposure to aflatoxins. As a general summary, AFB<sub>1</sub> lowers lymphoid cell populations especially circulating activated lymphocytes, suppresses lymphoblastogenesis and decreases both cutaneous delayed-type hypersensitivity (DTH) and graft versus host reaction (Meissonnier *et al.*, 2008). AFB<sub>1</sub> reduces natural killer cytotoxicity and macrophage functions including phagocytic activity and production of oxidative radicals. A brief literature review is provided for the immunotoxic effects of aflatoxins in different species.

The immunotoxic effects of aflatoxins have been studied in pigs. Treatment with AFB<sub>1</sub> has been shown to shorten the incubation period of *Serpulina* (*Treponema*) *hyodysenteriae* in pigs (Joens *et al.*, 1981). Pigs given AFB<sub>1</sub> and infected with *S. hyodysenteriae* also had a longer period of diarrhea. Feeding weanling piglets a diet containing 140 or 280 ppb AFs (~70% AFB<sub>1</sub>) for 4 weeks caused an increase in serum  $\gamma$ -globulin (Marin *et al.*, 2002). At the 280 ppb level, a reduced immune response to *Mycoplasma agalactiae* was observed. There were indications that interleukin-1 $\beta$  and tissue necrosis factor- $\alpha$  expression was decreased by exposure at the 280 ppb level. Sows were fed diets containing 800 ppb AFB<sub>1</sub> or

a diet containing 400 ppb AFB<sub>1</sub> + 400 ppb AFG<sub>1</sub> for the entire gestation and lactation period (Silvotti *et al.*, 1997). At 25 days of age the piglets born to the sows were found to have decreased immune functions in lymphoproliferative response to mitogens, monocyte-derived macrophages failed to have induced oxygen bursts and granulocytes had reduced chemotactic response. There is some evidence that *in utero* exposure of piglets to AFB<sub>1</sub> interferes with zinc metabolism and low plasma zinc decreases cell-mediated immunity because of a decrease in a zinc-dependent thymic hormone (Mocchegiani *et al.*, 1998).

Aflatoxins have been shown to be immunotoxic in ruminants. Feeding a diet containing 2 ppm AFs (83.4% AFB<sub>1</sub>, 12.3% AFB<sub>2</sub> and AFG<sub>1</sub> and AFG<sub>2</sub>) to lambs for 37 days increased serum  $\gamma$ -globulin, decreased bacteriostatic activity in serum and decreased cellular immunity (Fernandez *et al.*, 2000). Studies done *in vitro* have shown that aflatoxin inhibits thymidine uptake by phytohemagglutinin-stimulated lymphocytes (Bodine *et al.*, 1984). Holstein steers (183 kg) fed a diet containing ~375 ppb aflatoxin for 17.5 weeks were observed to have a decrease in delayed cutaneous hypersensitivity (Richard *et al.*, 1983). Changes were not observed in antibody production (*Brucella abortus* antigen) and lymphoblastogenesis. Cattle were administered 0.3 mg of AFB<sub>1</sub> equivalent/kg body weight for 12 to 14 days (Brown *et al.*, 1981). Before administration of aflatoxins, the cows were previously infected intramammary with *Streptococcus agalactiae*, *Staphylococcus aureus* and *Staphylococcus hyicus*. Clinical evidence of mastitis was not observed, but three of four cows had increased bacteria in the milk. The California mastitis test scores increased after the last dose of aflatoxins were given. Steers were administered a single oral dose of 0.6 or 0.8 mg AFB<sub>1</sub>/kg body weight (Thurston *et al.*, 1986). Complement activity and bacteriostatic activity of serum decreased for 48 to 72 h and then returned to baseline at 7 days. In the same study cattle were administered 0.25 mg AFB<sub>1</sub>/kg body weight/day for 14 days. The bacteriostatic activity of serum decreased until day 11 and then remained decreased for 29 days.

The immunotoxic effects of aflatoxins have been studied in poultry. Immunotoxic effects in poultry are lymphocytopenia, reduced T cell counts and reduced native resistance to disease (Pier and Heddleston, 1970). Two-week-old turkey poults were placed on a diet containing 50 and 150 ppb aflatoxins for 11 weeks (Richard *et al.*, 1986). The poults were vaccinated with sheep red blood cells (SRBC) or *Pasteurella multocida* at 4 weeks and the vaccinations repeated at 8 weeks. Poults in the 150 ppb group had an increase in humoral response to *P. multocida* at 4 weeks and a decreased response to SRBC at 8 weeks. Three-week-old chicks were injected intramuscularly with 0.250 mg AFB<sub>1</sub> every 2 days for

four administrations of AFB<sub>1</sub> (Otim *et al.*, 2005). At 3 and 6 weeks of age the chicks were vaccinated with Newcastle virus vaccines. Hemagglutination reaction to Newcastle disease virus was reduced. The immunosuppression of aflatoxins (from cultures of *A. flavus* NRRL 5518) were found to be additive with fowl adenovirus serotype-4 (Shivachandra *et al.*, 2003). The response to dinitrochlorobenzene hypersensitization was also reduced by AFB<sub>1</sub>. Hens were fed diets containing 0.2, 1.0 and 5.0 ppm AFB<sub>1</sub> and fertile eggs were collected (Qureshi *et al.*, 1998). All chicks from hens given aflatoxins had reduced macrophage phagocytic activity and reactive oxygen burst. Antibody titers to SRBC were not changed on the primary vaccination, but the secondary response was depressed in chicks from hens given the diet containing AFB<sub>1</sub>. A study by Sur and Celik (2003) showed that decreased development of the bursa of Fabricius was observed in chicks after the egg was injected with 10 µg AFB<sub>1</sub> at day 7 of incubation.

## Reproductive and teratogenic effects

The reproductive effects of aflatoxins are best described as aflatoxin-linked deviation from the expected reproductive outcome. Two recent reviews have been published (Shuaib *et al.*, 2010a; Gupta, 2011). The reproductive effects in poultry have been described (Ortatli *et al.*, 2002). Feeding AFs, which had been previously produced in wheat using *A. parasiticus*, to mature roosters at 0, 2.5, 10 and 20 ppm AFs for 8 weeks caused testicular degeneration and a reduction in plasma testosterone. It is important to point out that toxicants that target spermatogenesis and spermiogenesis may not be apparent for 3 to 6 weeks, dependent on the species, due to the time required for spermiogenesis. A study in chickens using aflatoxins produced in rice by *A. parasiticus* showed that aflatoxins fed at 0, 10 and 20 ppm led to a reduction in testicular mass at 122 weeks of age and a reduction in plasma luteinizing hormone (Clarke and Ottinger, 1987). This effect of aflatoxins appears to be a reduction in pituitary response to luteinizing hormone-releasing hormone (LHRH) (Clarke and Ottinger, 1987). The effects of aflatoxin on egg production, fertility, hatchability and progeny performance were investigated in mature chicken hens by feeding dietary aflatoxin produced by *A. parasiticus* produced in rice culture (Howarth and Wyatt, 1976). Rice culture was added to the diets to give dose levels of 0, 5 and 10 µg aflatoxins/g for 4 weeks. Feeding aflatoxins significantly decreased egg production at 3 and 4 weeks. Egg fertility was not affected by dietary aflatoxins, but hatchability decreased significantly within 1 week. At the third week, chicks from hens on the 10 ppm aflatoxins diet had decreased body mass at hatching. An Egyptian study feeding 100 ppm aflatoxins to mature Mamourah hens

for 6 weeks showed that the hatch weight of the chicks was higher than controls (Abdelhamid and Dorra, 1990). Aflatoxins have been shown to be disruptive of sperm production and fertility in mice. An intraperitoneal dose of 50 µg of AFB<sub>1</sub>/kg body weight/day (estimated to be equivalent to ~330 ppm in diet) was given to male mice for varying intervals (Agnes and Akbarsha, 2003). At 35 days, fertility testing showed a decrease in litter size, and tissue examination showed a decrease in spermatozoa numbers present in the caudal epididymis. When the numbers of spermatozoa decreased, forward mobility of spermatozoa was decreased and abnormal spermatozoa were observed.

The effects of aflatoxins on human reproduction have been reviewed (Shuaib *et al.*, 2010b). There is evidence that dietary aflatoxins decrease male fertility (Ibeh *et al.*, 1994). A study at an infertility clinic showed that there is a correlation between dietary levels and serum levels of aflatoxins. The mean aflatoxin levels in serum were  $1.660 \pm 0.04$  µg/ml (infertile men) and  $1.041 \pm 0.01$  µg/ml (fertile men). The infertile men had a higher percentage (50%) of abnormal spermatozoa and aflatoxin in their semen. Exposure to AFs decreased human fertility by increasing fetal loss (Gupta, 2011). Women with high serum levels of AFB<sub>1</sub>-lysine adduct ( $\geq 11.34$  pg/mg of albumen) are at greater risk for delivering a child with low birth weight. There is a trend for delivery of low birth weight babies with increasing levels of AFB<sub>1</sub>-lysine adduct. Women with increased blood levels of AFM<sub>1</sub> had increased risk to give birth to a baby with abnormally low birth weight (Abdulrazzaq *et al.*, 2002).

Fetal loss and possible teratogenic effects have been reported for AFs. Sows were fed a diet containing 800 ppb AFB<sub>1</sub> or 400 ppb AFB<sub>1</sub> + 400 ppb AFG<sub>1</sub> from day 60 of pregnancy to day 28 of lactation (Mocchegiani *et al.*, 1998). Pigs born to the sows on the 800 ppb AFB<sub>1</sub> diet had reduced birth weights. Reduced birth weight was more pronounced in piglets born to sows consuming the diet containing 400 ppb AFB<sub>1</sub> + 400 ppb AFG<sub>1</sub>. Pregnant rats were administered 0.3 mg of AFB<sub>1</sub>/kg body weight on gestation days 11 to 14 or gestation days 15 to 18 (Kihara *et al.*, 2000). The offspring of these rats showed that prenatal exposure to AFB<sub>1</sub> caused a delay in early response development, decreased learning ability and impaired locomotor coordination. The effects of aflatoxins on weight gains have been modeled using previously published studies (Dersjant-Li *et al.*, 2003).

## CLINICOPATHOLOGY

### Introduction

Acute aflatoxicosis causes changes in clinicopathology parameters. The activities of liver enzymes in serum

are increased and the increased activity is indicative of hepatic damage. Increased activity of hepatic enzymes in serum include  $\gamma$ -glutamyl transpeptidase (transferrase) (GGT), aspartate aminotransferase (AST, previously identified as SGOT), alkaline phosphates (ALP), sorbitol dehydrogenase (SDH), ornithine carbamyl transferase (OCT) and isocitric dehydrogenase (ICD). Once released into serum the activities of hepatic enzymes decrease as metabolic processes remove the hepatic enzyme from serum. The activities of hepatic enzymes in serum that have been reported to be the most consistently elevated in aflatoxicosis are GGT, AST and SDH. The elevated activity of ALP is variable in aflatoxicosis. The timing of hepatic enzyme determinations in terms of the pathogenesis of aflatoxicosis is important and enzymatic activity must be interpreted in the context of the temporal aspect of aflatoxicosis. The activity of GGT has a slower temporal decrease than AST. Research studies comparing treatment to control animal can have statistically significant increases in clinicopathology parameters that are inside the expected normal range for the species. The interactions with duration of exposure can be statistically significant. Serum chemistry is useful in the diagnosis of aflatoxicosis. Prothrombin time is generally increased and frank hemorrhage can occur. Serum bilirubin levels are also increased and photosensitization can occur. The changes in hematological parameters generally are due to hemoconcentration and blood loss.

## Cattle

Steers were given single oral doses (0.2, 0.4, 0.6 and 0.8 mg/kg body weight) of aflatoxins (Cook *et al.*, 1986). Increases in the serum activity of SDH and AST and not ALP were observed. The increase started at 8 h after dosing and reached maximal activity at 24 to 48 h and then rapidly decreased. Brucato *et al.* (1986) administered a single oral dose of 1 mg AFB<sub>1</sub>/kg body weight to calves. Serum AST and GGT activities were increased within 2 days. After 2 days, the AST activity rapidly decreased whereas the GGT activity was elevated for 3 weeks. Aflatoxicosis from consuming high levels of aflatoxins in a concentrate has occurred in cattle with an increase in serum activity of AST and no increase in GGT activity (Crockcroft, 1995). Feedlot steers fed a diet containing 600 ppb AFB<sub>1</sub> for 155 days had an increase in AST, ALP and SDH (Helferich *et al.*, 1986). Steers fed diets containing 60 and 300 ppm AFB<sub>1</sub> did not have an increase in AST, ALP and SDH. Holstein calves (70 kg) were fed a diet containing 5 ppm aflatoxins for 3 weeks (Wyatt *et al.*, 1985). Serum activity of lactic acid dehydrogenase (LDH) decreased, ALP peaked on treatment day 7 and then decreased, and AST increased to study day 14 and then plateaued. Holstein steers (183 kg) fed a diet containing ~375 ppb aflatoxin for 17.5 weeks were observed to

have an increase in SDH and a decrease in AST activities (Richard *et al.*, 1983). Vaid *et al.* (1981) reported chronic aflatoxicosis in cattle consuming AFB<sub>1</sub> (0.11 ppm) contaminated groundnut cake. Hepatic enzymes in serum with increased activity were ALP and AST in one of four animals sampled. Timing of the blood sampling in terms of aflatoxicosis was not given, and these animals had histopathology consistent with aflatoxicosis. A study by Lynch *et al.* (1970) in calves suggested that the threshold dose for AFB<sub>1</sub> to increase AP was 0.1 mg/kg body weight fed for 6 weeks.

## Sheep and goats

Goats given 3 mg of AFB<sub>1</sub>/kg body weight by intramuscular injections had increased serum activity of AST which peaked at 32 to 40 h and then decreased (Hatch *et al.*, 1982; Clark *et al.*, 1984). Sheep fed mold-damaged wheat that contained an estimated 50 ppb AFB<sub>1</sub> had a linear decrease in total serum protein, serum albumin and serum globulins (Tripathi *et al.*, 2008). Serum urea nitrogen and glucose increased, AST slowly increased with the duration of feeding and IgG decreased. Histopathology was not done and the feed was not assayed for other mycotoxins. Lambs fed aflatoxins produced from a culture of *A. parasiticus* for 37 days had decreased feed intake and average daily gains did not have a decrease in WBC and RBC numbers (Fernandez *et al.*, 2000). Parameters assessing immune function were decreased. Wethers did not have elevated serum activity of AST and GGT and hematology parameters after consuming groundnut cake containing an estimated level of 750 ppb AFB<sub>1</sub> (Suliman *et al.*, 1987). Hepatic necrosis and other hepatic and renal lesions were observed. The timing of clinicopathologic determinations with the onset of clinical signs was not given. Sheep given 0.1 mg of AB<sub>1</sub>/kg body weight for 34 days had decreased albumin, increased  $\gamma$ -globulins and decreased  $\beta$ -globulins (Miller *et al.*, 1984).

## Swine

Growing barrows were fed a diet containing 1, 2, 3 or 4 ppm aflatoxins for 28 days (Harvey *et al.*, 1988). On day 14, serum activity of AST and GGT increased in pigs fed the 4 ppm diet, on day 21 ALP increased in barrows fed the 4 ppm diet and AST increased in all pigs fed aflatoxins, and on day 28 ALP activity was increased in pigs fed aflatoxins, GGT was increased in pigs fed the 3 and 4 ppm diets and serum AST activity was increased in pigs fed the 4 ppm diet. Barrows were fed diets containing 0, 250 and 500  $\mu$ g AFB<sub>1</sub>/kg of feed (Rustemeyer *et al.*, 2010). The source of AFB<sub>1</sub> was rice culture of *A. parasiticus* and the whole culture material was added

to the finished ration. When compared to controls AST was increased, and at 5 weeks of exposure BUN was increased. Minimal histopathology (mild biliary hyperplasia and nuclear pleomorphism) was observed. At both treatment levels, changes were observed in performance parameters.

## Dogs

Dogs (1.5 years old) administered intravenously 15 doses of 0.05mg AFB<sub>1</sub>/kg body weight for 15 days had increased serum activity of ALP and ICD (Chaffee *et al.*, 1969). Nine dogs that had consumed commercial dog food containing aflatoxin-adulterated corn were evaluated by clinicopathology parameters (Newman *et al.*, 2007). Findings were 5/8 with elevated WBC numbers, 6/9 with decreased platelets, 7/7 with increased prothrombin and partial thromboplastin times, 6/7 with decreased total serum protein, 7/7 with decreased serum albumen, 6/8 with decreased serum globulin, 7/9 with elevated ALP, 9/9 with elevated ALT, 6/6 with elevated AST, and 9/9 with elevated total bilirubin. Aflatoxin levels in the finished dog food were from ~223 to 579ppb AFB<sub>1</sub> and 16 to 10ppb AFB<sub>2</sub>. AFM<sub>1</sub> levels in the liver ranged from 1.20 to 4.40ppb. Dogs have died from aflatoxicosis in human outbreaks (Wild and Gong, 2010).

## Horses

The clinicopathology of aflatoxicosis has been studied in horses. Weanling ponies were administered AFB<sub>1</sub> at 0.0, 0.5, 1.0 and 2.0mg/kg body weight (Boatell *et al.*, 1983). Serum activity of GGT was increased in all ponies administered AFB<sub>1</sub>. The GGT activity increased until day 3 and then decreased. Serum activity of ALT was not increased. Ponies given 4, 5, 6 and 7.4mg AFB<sub>1</sub>/kg body had an increase in serum ALT and the activity of ALT increased until the ponies died at 33 to 46h after dosing.

## Poultry

Clinicopathology has been described for aflatoxicosis in poultry (Hashem and Mohamed, 2009), with decreased erythrocyte numbers, hemoglobin, leukopenia consisting of heteropenia and lymphopenia. Elevations occurred in ALT, AST, GGT and ALP, uric acid and creatine. Total serum protein, globulins and albumin were also decreased. Wild turkey poults were fed diets amended with rice culture (*A. parasiticus*) to give 0, 100, 200 and 400ppb aflatoxin/kg of feed (Quist *et al.*, 2000). The poults were 4 months old at the start of the study which

lasted for 14 days. Trends were observed for treated birds to have an increase in heterophil and lymphocyte numbers, and a decrease in basophil numbers. Serum AST was increased, and triglycerides and prothrombin time had a trend to decrease.

## Humans

Aflatoxicosis in humans has been reported. It is estimated that an intake >20µg/kg body weight/day is fatal for humans and deaths can occur within 7 to 21 days (Wild and Gong, 2010). Studies on reproduction have been done in humans endemically exposed to aflatoxins. Pregnant women with lower socio-economic status have higher risk for increased AFB<sub>1</sub>-lysine adduct in their serum (Shuaib *et al.*, 2010a). The levels of AFM<sub>1</sub> in cord blood of humans are correlated with the levels of AFM<sub>1</sub> in maternal blood (Abdulrazzaq *et al.*, 2002). The range of AFM<sub>1</sub> in maternal blood was 0.05 to 10.44ng/ml and the range of AFM<sub>1</sub> in cord blood was 0.03 to 8.49ng/ml. Other authors have postulated that a reduction of maternal AF-lysine adducts from 110pg/mg of albumin to 10pg/mg of albumen would give a 0.8kg increase in body mass and a 2cm increase in height during the first year of life (Turner *et al.*, 2007).

## MACROSCOPIC AND MICROSCOPIC PATHOLOGY

### Edema

The pathology of aflatoxicosis has been described in several species. Edema including anasarca and edema fluid in the peritoneal and thoracic cavities can be observed (Osweiler and Trampel, 1985; Bastianello *et al.*, 1987; Coppock *et al.*, 1989; Hall *et al.*, 1989). The gall bladder, bile duct, abomasal wall and colonic mesentery may be edematous. There may be edema of the omentum near the stomach or abomasum and in the colonic mesentery. Gall bladder edema may be marked in cattle and pigs. Edema of the brain has been reported for horses (Angsubhakorn *et al.*, 1981).

### Hemorrhage

Hemorrhage into the gastrointestinal tract, into body cavities and on body organs has been reported. Hemorrhage is due to decreased production of clotting factors by the liver. Acute aflatoxicosis in cattle, horses, pigs and dogs may result in serious hemorrhages in the gastrointestinal tract, on serosal surfaces, on the



epicardium and endocardium, in skeletal muscle, perirenal and in the urinary bladder (Cysewski *et al.*, 1968; McKenzie *et al.*, 1981; Boatel *et al.*, 1983; Jakhar and Sadana, 2004). Pigs can also have hemorrhage occurring in the pleural and peritoneal cavity. The urine may be dark red colored.

## Hepatic lesions

Histopathology includes bile duct proliferation, hepatocyte necrosis and early fibrosis of the liver (Newberne and Butler, 1969). Regeneration of hepatocytes may be found in dogs, and nodular hyperplasia may be present in turkeys, trout and ducklings (Newberne *et al.*, 1966b; Newberne and Butler, 1969; Bastianello *et al.*, 1987). Variation in the size of hepatocytes, with some megacocytes and binucleate cells, is recorded in many reports. Chronic liver lesions are characterized by firm, fibrous pale livers, by nodular hyperplasia, and in some cases neoplasia. Icterus is present. The liver may be swollen, friable and congested. Histologically, the liver may have necrosis in variable patterns. In the rat, monkey and duckling, the necrosis is reported to be periportal, while in cattle, pigs, horses, goats and sheep, the pattern is recorded to be centrilobular (Butler, 1964; Newberne *et al.*, 1966a; Samarajeewa *et al.*, 1975; Carlton and Szczech, 1978; Abdelsalam *et al.*, 1989; Coppock *et al.*, 1989; Mathur *et al.*, 1991). In the dog the pattern may be either or both periportal and centrilobular, while in the rabbit the pattern is reported to be midzonal (Newberne *et al.*, 1966a; Chaffee *et al.*, 1969; Clark *et al.*, 1980). Hyperplasia of the bile duct cells occurs rapidly in ducklings and may be present in horses, dogs and chickens, and mild bile duct cell hyperplasia may be seen in cattle and pigs (Newberne and Butler, 1969; Ketterer *et al.*, 1975; Coppock *et al.*, 1989). Multinucleated hepatocytes have been observed in dogs, cattle and other species. Reactive fibroblasts have been observed in dogs. Humans infected with hepatitis B virus appear to be at greater risk for hepatic neoplasia when they are exposed to aflatoxins (Wild and Gong, 2010). Chronic liver injury and regenerative hyperplasia are considered critical for the development of liver neoplasia. Dividing liver cells are more likely to form DNA adducts. In sub-Saharan Africa, consumption of corn is associated with increased HIV and consumption of rice is associated with increased liver cancer (Williams *et al.*, 2010). Hepatic encephalopathy could occur.

## Other lesions

Renal lesions of protein in proximal tubules and glomerular spaces are recorded in horses and rats (Butler,

1964; Newberne and Butler, 1969; Boatell *et al.*, 1983). Hemosiderin deposition in tubule cells occurs in horses (Angsubhakorn *et al.*, 1981). Cardiac myofiber degeneration is recorded in horses and rats (Butler, 1964; Angsubhakorn *et al.*, 1981). Brain lesions described as focal malacia in the cerebral hemispheres in horses were reported (Angsubhakorn *et al.*, 1981). Focal malacia has not been reported by others or in other species. However, hepatic encephalopathy could occur as a result of liver damage. Subacute lesions observed at necropsy include firm, pale livers in all species and clear yellow ascites and pleural fluid accumulation especially in pigs and dogs (Newberne and Butler, 1969). Icterus is usually present. Edema of the mesentery near the colon and perirenal edema may be present in pigs and cattle (Osweiler and Trampel, 1985; Bastianello *et al.*, 1987; Coppock *et al.*, 1989; Hall *et al.*, 1989). Pulmonary edema may occur. Pneumonia is reported in calves as likely secondary to edema and the effects of aflatoxin on the immune system. Lesions of photosensitization have been reported in cattle.

## DIFFERENTIAL DIAGNOSIS

The lesions of aflatoxicosis and those of pyrrolizidine alkaloid toxicosis have been observed to be very similar especially in cattle (Loosmore and Markson, 1961). Cattle are generally considered to be more susceptible to pyrrolizidine alkaloids. When aflatoxins and pyrrolizidine alkaloid are at issue, it is important to examine pasture, forage, concentrate feeds and gastrointestinal contents to determine the presence of poisonous plants that may contain pyrrolizidine alkaloids. In some geographic areas seeds containing pyrrolizidine alkaloids can be present in grain screenings, and their identity lost in the grinding and pelleting processes. It is the experience of the authors that domestic animals with signs of aflatoxicosis never fully recover.

## TREATMENT AND OUTCOMES

A specific treatment for aflatoxicosis has not been identified. Aflatoxins from all sources should be removed from the diet. Symptomatic care of the affected animals is recommended. Animals on a low protein diet are more susceptible to aflatoxins (Cullen and Newberne, 1994). Supplementing diets with choline and methionine and N-acetylcystine is probably beneficial (Cullen and Newberne, 1994; Valdivia *et al.*, 2001). Adding vitamin E to swine diets containing 2.5ppm aflatoxins did not

have a protective effect on the severity of aflatoxicosis (Harvey *et al.*, 1994). Vitamin E was observed to have an ameliorative effect on the adverse effects of AFs on weight gain in hens, but did not prevent the pathologic effects in liver and kidney (Kahn *et al.*, 2010). Increasing dietary zinc did not appear to be of benefit to calves fed a diet containing 5 ppm aflatoxins (Neathery *et al.*, 1980). There is some suggestion that selenium provided some protection to Japanese quail fed a diet containing 1 ppm AFB<sub>1</sub> (Jakhar and Sadana, 2004). Excessive dietary selenium increases the toxicity of aflatoxins. Pimpukdee (2004) found that a proprietary alumino-silicate (feed anti-caking agent) protected chicks from hepatic loss of vitamin A linked to aflatoxins in the diet. Feeding sodium bentonite was considered protective to pigs (Schell *et al.*, 1993). Diaz *et al.* (2004) showed that sodium bentonite and MTB-100 (derived from the cell wall of yeast) reduced AFM<sub>1</sub> in milk.

## PREVENTION OF AFLATOXINS

Avoidance of formation and consumption of AFs is the best prevention (Mishra and Das, 2003). Different materials have been shown to reduce the toxic effects of AFs. Green tea, likely polyphenols, reduces or blocks initiation of AFB<sub>1</sub>-induced hepatocarcinogenesis. Enteric binding of AFs has been studied (Phillips *et al.*, 2008). Studies have shown that clays in the smectite group, including hydrated sodium calcium aluminosilicate (calcium montmorillonite), reduce the toxicity of dietary aflatoxins in a number of species. The smectite clay in the gut adsorbs the AFs making them biologically unavailable. The surface activity of the smectite clay appears to have a unique affinity for AFs. Studies in Africa showed that ingestion of a heat-processed montmorillonite clay reduced aflatoxin adducts in blood and excretion of AFM<sub>1</sub> in urine (Wang *et al.*, 2008). It is of interest to point out that clay eating has been observed to be a long-standing practice in some African societies. Modified yeast cell wall *Saccharomyces cerevisiae* can decrease absorption of AFs in some species and not in others with the results being variable (Battacone *et al.*, 2009; Firmin *et al.*, 2010).

## AFLATOXIN INTERACTIONS

Multiple mycotoxins can occur when different feed ingredients are blended to manufacture a finished feed, or a feed or feed ingredient has supported the growth of a succession of fungi. When one mycotoxin is found

in feedstuffs, the toxicologist should consider that additional mycotoxins of a different group could be present (Huff *et al.*, 1988). The presence of multiple mycotoxins can alter the dose-response curve. Therein, the level of a mycotoxin that is generally considered to be safe can be altered by the presence of other mycotoxins. The interactions of mycotoxins may vary between the indicators of effect. The interactions could be additive for one parameter and synergistic for another. A feeding study in mice showed that a combination of fumonisin B<sub>1</sub> (FB<sub>1</sub>) at 10 ppm and AFB<sub>1</sub> at 10 ppb increased feed consumption and decreased weight gains over a 90-day study interval (Casado *et al.*, 2001). Liver lesions were considered to be intensified by the combination and this effect increased temporally. AFB<sub>1</sub> and FB<sub>1</sub> combination were shown to decrease feed conversion and feed consumption in feeder pigs (Dilkin *et al.*, 2003). The dietary levels were 30 ppm FB<sub>1</sub> and 50 ppb AFB<sub>1</sub>. Pigs were fed a diet containing aflatoxins produced in rice culture, a diet containing FB<sub>1</sub> produced in corn culture and a diet containing both the rice and corn cultures (Harvey *et al.*, 1995). The interactions of aflatoxins and FB<sub>1</sub> for immunologic and liver disease parameters were essentially additive. Cultures containing aflatoxins and T<sub>2</sub> toxin when fed in combination to Japanese quail showed interactions appeared to be additive (Madheswaran *et al.*, 2004). Increased abnormal chicks were observed when ochratoxin A (2 µg) and AFB<sub>1</sub> (0.5 µg) were injected into eggs on day 3 of incubation (Edrington *et al.*, 1995). Calves were fed diets containing ochratoxin A and AFB<sub>1</sub> (Patterson *et al.*, 1981). The sources of the mycotoxins were naturally contaminated barley or naturally contaminated groundnut meal. The dose of ochratoxin A was 390 to 540 µg/kg body weight and the dose of AFB<sub>1</sub> was 10 to 13 µg/kg body weight. No adverse health effects were observed when the toxins were fed individually or as a mixture, possibly because of the ability of rumen organisms to degrade the mycotoxins. The interactions of AFs and deoxynivalenol (DON) in naturally contaminated maize were studied in pigs (Chaytor *et al.*, 2011). This study showed that diets containing both AFs and DON greater than 60 and 300 µg/kg, respectively, may diminish growth and reduce feed intake. Diets containing 120 µg of AFs/kg and 600 µg of DON/kg may reduce native resistance to disease, alter systemic inflammation and cause liver damage.

Preexisting disease can increase susceptibility to aflatoxins. Osuna *et al.* (1977) concluded that preexisting liver flukes increased the susceptibility of cattle to aflatoxins and the interactive effect was additive. Henry *et al.* (2002) estimated that the presence of hepatitis B surface antigen in serum increased the human susceptibility to aflatoxins by a factor of 30. Shivachandra (2003) showed the immunosuppression of aflatoxins was additive with fowl adenovirus serotype-4. Ethanol was

found to potentiate AFB<sub>1</sub>-induced hepatocarcinogenesis (Tanaka *et al.*, 1989).

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

Aflatoxins, on a world scale, are one of the most important groups of mycotoxins (Lanyasunya *et al.*, 2005). Aflatoxins target the liver immune system, kidney and other body organs. Aflatoxin B<sub>1</sub> is the most potent known naturally occurring carcinogen. Metabolism of AFs varies between species and likely explains the species differences in toxicity. Reactive metabolites of AFs form adducts with nucleic acids and proteins. Human and animal populations are exposed to AFs in foodstuffs and feedstuffs, respectively. The parent aflatoxin and its toxic metabolites can be present in edible animal products. The interactive effects of aflatoxins with infectious and parasitic diseases and other toxins have been demonstrated. Agronomic practices, pest control and ensuring that stored commodities cannot support growth of fungi are the best control for prevention of aflatoxin contamination of foods and feeds.

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# Ergot

Steven S. Nicholson

## INTRODUCTION

Ergot is a general term that applies to all species of the fungus *Claviceps*. Ergotism refers to the disease conditions associated with ingestion of toxic levels of ergot by animals and humans. Ergot contaminated rye (*Secale cereale*) used as flour was responsible for episodes of human ergotism in Europe during the Middle Ages. Significance, chemistry and determination of ergot alkaloids were reviewed recently by Krska and Crews (2008). The genus *Claviceps* includes very specialized fungi which parasitize the flowers of grasses and cereal grains with no other part of the plant infected. Recently, ergot alkaloids produced by *Claviceps cyperi* in nut sedge were described as a toxicant in dairy cattle (Naude *et al.*, 2005). *Claviceps africana* ergot in sorghum produces dihydroergosine (DHES) and related alkaloids, which cause hyperthermia in cattle but dilution in ensilage reduced average concentrations of DHES to approximately 1 mg/kg, a relatively safe level for cattle (Blaney *et al.*, 2010). This chapter primarily addresses alkaloids produced by *Claviceps purpurea* and the various toxic effects, called ergotism, which they produce in animals and humans. The source of exposure to animals includes ergot-infected grains in feeds such as barley, rye, wheat and oats as well as infected seeds in forages consumed while grazing or in hay and silage. Removal of ergotized seeds along with broken grain kernels and debris by the process of mechanical screening of harvested and stored grain concentrates the ergot in the screenings. Utilizing such contaminated grain screenings for human food or animal feed would create a distinct hazard.

Clinical syndromes include gangrene of the extremities of cattle and horses, hyperthermia and production loss in cattle, agalactia and abortion in swine, agalactia

and reproductive effects in mares. Most countries have a regulatory limit of 0.1–0.2% ergots in flour. The suggested maximum level for safety is 0.05%. In the U.S., wheat and rye with 0.3% sclerotia are considered unsafe and oats, triticale, or barley having more than 0.1% sclerotia are deemed unfit for human consumption.

Clinical disease associated with *Claviceps* has a parallel in fescue grass toxicosis (see Chapter 87) where gangrenous ergotism, hyperthermia, production loss in cattle and adverse effects on reproduction and lactation in horses are similar (Evans *et al.*, 2004). The seeds of fescue grass can be infected with *Claviceps purpurea* but the ergot alkaloids of fescue grass toxicosis are produced by the endophyte fungus *Neotyphodium coenophialum*. Ergot alkaloid-induced fescue toxicosis has been studied in much more detail than has ergotism from ingested *C. purpurea* sclerotia.

Ergovaline produced in perennial and annual ryegrasses by the endophyte fungus *Neotyphodium lolii* is associated with clinical toxicosis (Schneider *et al.*, 1996; Bourke, 2003).

## BACKGROUND

*C. purpurea* has a host range exceeding 200 species of grasses. It is distributed worldwide in temperate climatic zones. During infection by *C. purpurea*, the ovary of the grass seed or cereal grain is replaced by fungal mycelia. A sticky exudate known as honeydew is produced that contains conidia which can be transferred to infect other seeds. The honeydew hardens to form a hard brown, purple or black compact mass of fungal tissue called a sclerotium. The ergot bodies or sclerotia contain up to



1.2% dry weight of toxic alkaloids (Burrows and Tyrll, 2001). Sclerotia of most *Claviceps* species are one to four times larger than the host seed and are readily identified in cereal grains. Compared to sclerotia in oats or barley the sclerotia are quite small in grass seeds such as Bahia grass (*Paspalum notatum*). Sclerotia fall to the ground over winter and later complete the fungal life-cycle by germinating and producing ascospores capable of infecting the ovary of developing seeds.

Alkaloids from *C. purpurea* are among the most important natural products used by the pharmaceutical industry. Prior to the industrial cultivation of *C. purpurea* in pure culture, ergot was grown as a crop on rye (*Secale cereale*) under field conditions for use in the manufacture of important medicinal drugs. Synthesized ergot alkaloids have a variety of uses in human and veterinary medicine. Chemical structures of some of the ergot alkaloids are shown in Figure 89.1.

## PHARMACOKINETICS/ TOXICOKINETICS

Ergot alkaloids responsible for most clinical signs and lesions are of the ergopeptine class including ergotamine, ergocristine, ergosine, ergocornine, ergocryptine and ergovaline (Cheeke, 1998; Carson, 1999; Burrows and Tyrll, 2001; Evans *et al.*, 2004). Injected intravenously, ergopeptine alkaloids are rapidly cleared from the blood by the liver (Cheeke, 1998) and excreted in the bile (Evans *et al.*, 2004). These alkaloids are not secreted in the milk of cows (Cheeke, 1998).

## MECHANISM OF ACTION

Ergot alkaloids have structures similar to the biogenic amines norepinephrine, serotonin and dopamine. Vasoconstriction is produced by an agonist activity and this effect varies with different vascular beds. Hyperthermia and uterine stimulation are other effects. The alkaloids are antagonistic to dopamine at D<sub>1</sub> vasodilatory receptors. Dopaminergic activity at D<sub>2</sub> receptors causes inhibition of prolactin secretion (Goldstein *et al.*, 1980). The dopamine antagonist domperidone is an effective treatment in mares suffering agalactia and uterine effects induced by ergot alkaloids in endophyte-infected tall fescue grass (Redmond *et al.*, 1993).

## TOXICITY

Expression of clinical toxicosis caused by ergot alkaloids occurs in four forms in livestock. These include: cutaneous

and gangrenous lesions of the tail and extremities, hyperthermia and production loss, reproductive failure, and a convulsive or nervous form. The latter is apparently uncommon, not well documented and is perhaps caused by acute ingestion of a large dose of sclerotia. Muscle tremors and hyperexcitability are signs in cattle caused by non-ergot compounds produced by *Claviceps paspali* in seeds of paspalum grasses (Cole *et al.*, 1977). Staggers in horses grazing paspalum grass seeds infected with *Claviceps paspali* occur as well (Cawdell-Smith *et al.*, 2010). Indole-diterpenes and ergot alkaloids were confirmed in Bermuda grass (*Cynodon dactylon*) infected with *Claviceps cynodontis* from an outbreak of tremors in cattle in South Africa (Uhlig *et al.*, 2009). In countries where cereal grains in commerce are subject to regulatory oversight, the presence of ergotized seed at significant levels in food and feedstuffs is not common. Ergotism in cattle caused by sclerotia ingested while grazing remains a sporadic problem in the United States (Burrows and Tyrll, 2001).

The cutaneous and gangrenous form of ergotism is associated with subacute or chronic ingestion of the ergopeptine alkaloids. Cold temperatures enhance the clinical effects. Constriction of small arteries and arterioles leads to necrosis affecting all four limbs, tips of the ears and distal third of the tail. Distinct lines separating normal tissue from nonviable tissues appear. The odor of rotting flesh may be obvious and affected animals may continue to walk until sloughing of the digits occurs.

Hyperthermia was noted in steers exposed to sunlight and fed 180 mg/kg body weight of *C. purpurea* even at moderate ambient temperatures and humidity (Bourke, 2003).

Hyperthermia induced by high ambient temperature and humidity in lactating dairy cattle reduces feed intake and milk production, and increases embryonic losses. Ingestion of ergot alkaloids enhances this effect (Al-Tamimi *et al.*, 2003). Hyperthermia and a 30% drop in milk yield occurred in succession in two Holstein dairy herds (n = 240 and n = 150 milking cows, respectively) on the South African Highveld (Naude *et al.*, 2005). Examination of the maize silage from both farms revealed that it was heavily contaminated with nut sedge which contained minute sclerotia, identified as those of *Claviceps cyperi*. This was the first report of bovine ergotism not associated with the grass family Poaceae infected with *C. purpurea* or endophytes. High levels of total ergot alkaloids, predominantly ergocryptine, were found by LC-MS in the silage as well as in the total mixed ration (115–975 ppb and 65–300 ppb, respectively). The ergot alkaloid content (mainly ergocryptine) of the maize silage on the second affected farm was 875 ppb. A significant 4.6 liter decrease in milk production in a herd of Holstein-Friesian dairy cows in Australia was associated with high concentrations of

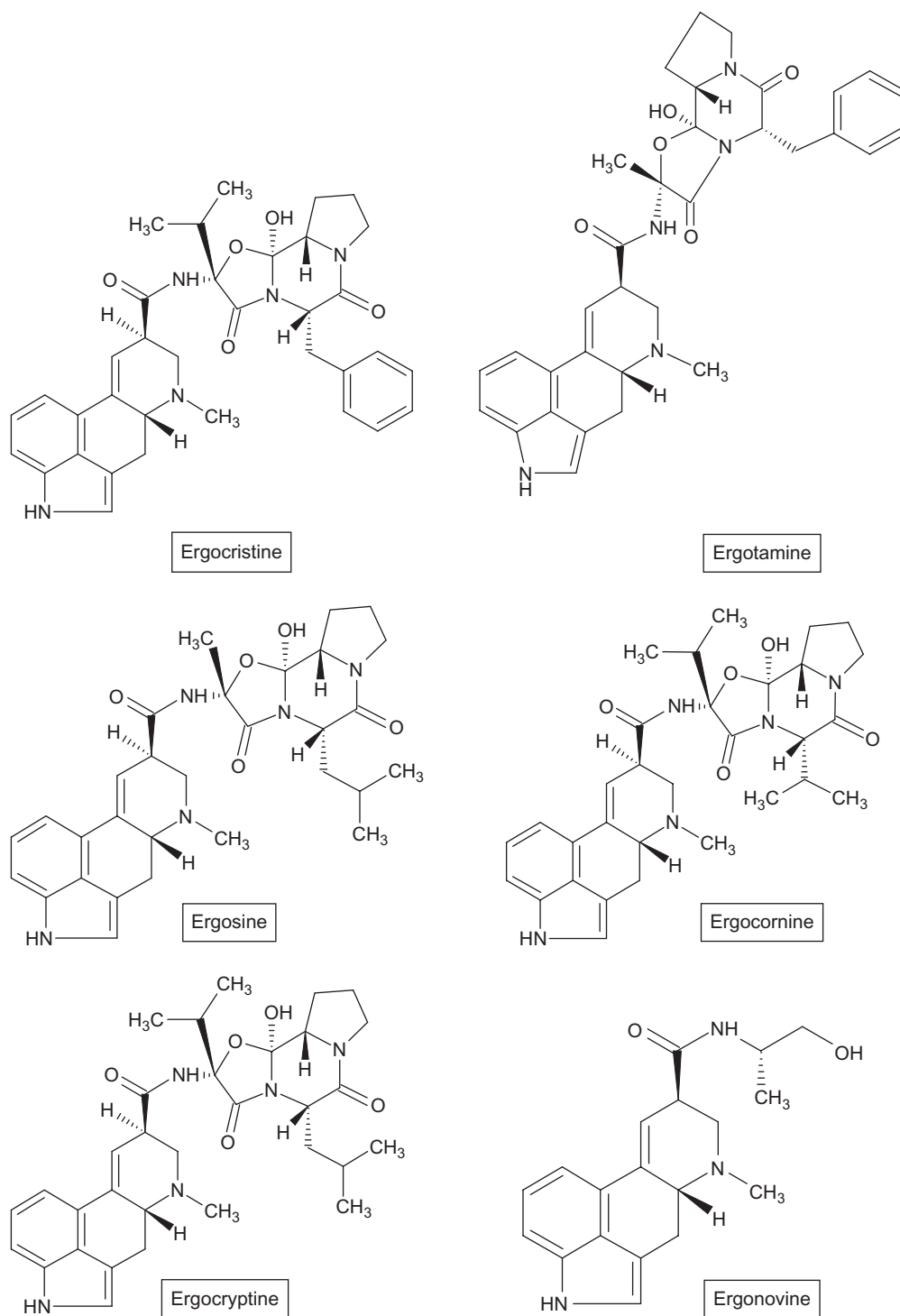


FIGURE 89.1 Chemical structures of some ergot alkaloids.

endophyte produced ergovaline ( $1.6\mu\text{g/g}$ ) in ryegrass silage (Lean, 2001). Simultaneously, milk somatic cell counts increased significantly and reproductive performance declined. Body condition score and coat condition of cows were adversely affected. In a study to determine if ergot alkaloids from *Claviceps purpurea* were carried

over to the milk, Holstein cows were fed contaminated diet (concentrate contained 2.25% ergot, which caused an alkaloid concentration of the daily ration between 504.9 and  $619.5\mu\text{g/kg DM}$ ) over a period of 4 weeks. Daily feed amounts were adjusted to the current performance which resulted in a dry matter intake variation between

6.0 and 18.5 kg/day. The actual alkaloid exposure varied between 4.1 and 16.3 µg/kg body weight when the ergot-contaminated concentrate was fed. Approximately 67% of the alkaloids fed were recovered in the duodenal ingesta, and approximately 24% were excreted with the feces. No alkaloid residues could be detected in the blood or milk samples (Schumann *et al.*, 2009).

Sheep are susceptible to the vasoconstrictive effects of ergopeptine alkaloids but ergot toxicosis is described as typically mild and not accompanied by gangrenous lesions (Greatorox and Mantle, 1973). Sloughing of the tip of the tongue is said to occur (Burrows and Tyrl, 2001).

The effects of ergot alkaloids in swine are primarily those of agalactia, production loss and an impact on reproduction. Diets containing 0, 1 and 10 g ergot (*C. purpurea*) per kg were fed to 12 pigs in the body weight range of 30–115 kg. Tendencies towards reduced feed intake and low rate of weight gain were observed at a feeding level of 4.66 mg total alkaloids per kg diet. At that level, protein digestibility was significantly reduced (Mainka *et al.*, 2005). Birth weight in pigs is reduced, stillborns increased and neonatal mortality increased due to agalactia in the sow (Lopez *et al.*, 1997).

Agalactia, small, weak foals, stillborn foals and placental edema occur in pregnant mares ingesting ergot alkaloids in the last month of gestation. This is a common occurrence in mares grazing endophyte-infected tall fescue grass in the United States. Similar effects caused by ingestion of *C. purpurea* sclerotia are seldom reported, perhaps because heavily infected ergot cereal grains are generally kept out of feed grain commerce. There remains the situation where heavily contaminated cereal grain screenings could be fed to horses. Twelve pregnant mares fed black oat (*Avena strigosa*) during the pre-delivery period experienced agalactia and delivered weak and unviable foals, which showed no suckling reflex and died within a few hours of birth. *C. purpurea* sclerotia were identified in 0.22% of the examined oat seeds (Copetti *et al.*, 2002).

Chickens develop gangrene involving the comb, beak and toes, weight loss and debilitation. Rabbits are extremely sensitive to the hyperthermic effects caused by ergot alkaloids (Burrows and Tyrl, 2001). Ergot alkaloids are embryocidal and abortifacient in laboratory animals (Mantle, 1969).

In dogs and rodents, corpora lutea are essential to maintain pregnancy. In these animals, ergot alkaloids inhibit prolactin secretion causing loss of corpora lutea and abortion.

Ergot alkaloids in oats fed to female mink bred to untreated males caused reduced numbers of kits born compared to controls (Sharma *et al.*, 2002). There was a significant effect on kit survivability with no kits surviving in the 12 ppm group. Serum prolactin was significantly depressed in the three ergot alkaloid groups compared to the control group. This study indicated that

ingestion of ergot alkaloids at 3 ppm or higher resulted in reproductive toxicity in mink.

## TREATMENT

Removing the source of ergot from the animals' diet is an obvious first step in treatment. The hyperthermic effect in dairy cattle should abate within 1 to 2 weeks. Animals that have developed gangrene of the extremities would not be expected to recover and should be euthanized.

The dopamine antagonist domperidone is an effective treatment in mares to prevent agalactia and uterine effects induced by ergot alkaloids in endophyte-infected tall fescue grass (Redmond *et al.*, 1993, 1995).

## CONCLUDING REMARKS

Ergot infection in cereal grains and grasses remains a concern for livestock and horses. Commercial cereal grain screenings and cereal grains produced and fed on the farm, and not inspected, are potential sources of ergotized grain. Exposure while grazing infected grasses may go unnoticed initially, especially if management is not aware of the risk.

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# Fumonisin

Geof W. Smith

## INTRODUCTION

Fumonisin ( $B_1$  and  $B_2$ ) are a group of naturally occurring mycotoxins produced by the fungus *Fusarium verticillioides* (formerly *F. moniliforme*). These toxic metabolites in corn have been implicated in field cases of porcine pulmonary edema (Harrison *et al.*, 1990; Osweiler *et al.*, 1992; Colvin *et al.*, 1993) and equine leukoencephalomalacia (Wilson *et al.*, 1990a). Experimentally, fumonisin has been shown to cause liver damage in multiple species including pigs, horses, cattle, rabbits and primates (Jaskiewicz *et al.*, 1987; Voss *et al.*, 1989; Haschek *et al.*, 1992; Osweiler *et al.*, 1993; Ross *et al.*, 1993; Gumprecht *et al.*, 1995) as well as species-specific target organ toxicity, such as lung in pigs (Haschek *et al.*, 1992), brain in horses (Ross *et al.*, 1993), kidney in rats, rabbits and sheep (Voss *et al.*, 1989; Edrington *et al.*, 1995; Gumprecht *et al.*, 1995) and esophagus in rats and pigs (Casteel *et al.*, 1993; Lim *et al.*, 1996). Epidemiologic data have linked ingestion of corn contaminated with *F. verticillioides* to human esophageal cancer (Rheeder *et al.*, 1992) and infant neural tube defects (Voss *et al.*, 2011); and fumonisins have been shown to be hepatocarcinogenic in rats and mice (Gelderblom *et al.*, 1988; Howard *et al.*, 2001).

## BACKGROUND

### Chemical structure

First isolated in 1988, the fumonisins are a group of structurally related compounds with the terminal carboxy group composed of propane-1,2,3-tricarboxylic

acid involved in ester formation with the C-14 and C-15 hydroxy groups. The 20 carbon chain base carries either 2-acetylamino or 2-amino-12,16-dimethyl-3, 5,10,14,15, pentahydroxyicosane (Figure 90.1). The structures of  $FB_1$  and  $FB_2$  have been shown to have the empirical formulas of  $C_{34}H_{59}NO_{15}$  and  $C_{34}H_{59}NO_{14}$ , respectively, with the only difference being the hydroxyl group present at the C-10 position in  $FB_1$  (Bezuidenhout *et al.*, 1988). Several additional fumonisin metabolites have been isolated ( $B_3$ ,  $B_4$ ,  $B_5$ ,  $B_6$ ,  $A_1$  and  $A_2$ ), but appear to occur in much lower concentrations than  $FB_1$  or  $FB_2$ , and are not considered important at this time (Gelderblom *et al.*, 1992; Mansson *et al.*, 2010). More recently, fumonisins have also been found in grapes and wine associated with the fungus *Aspergillus niger* (Mansson *et al.*, 2010; Mogensen *et al.*, 2010).

### Occurrence and distribution

Apart from reports of fumonisin  $B_1$  and  $B_2$  in “black oats” feed from Brazil (Sydenham *et al.*, 1992), and in New Zealand forage grasses (Mirocha *et al.*, 1992), the only commodities in which fumonisins have been detected so far are corn and corn-based foods. The occurrence of  $FB_1$  in the forage grass was accompanied by mono- and dimethyl esters which may or may not have been artifacts (Mirocha *et al.*, 1992). Equine leukoencephalomalacia (ELEM) has long been associated with the consumption of moldy corn, and has been reported in many areas of the world. However, cases of ELEM that were directly associated with fumonisin-contaminated feed have been reported in South Africa and Egypt (Thiel *et al.*, 1991), the United States (Wilson *et al.*, 1990a;

Ross *et al.*, 1991), Brazil (Sydenham *et al.*, 1992), Hungary (Bela and Endre, 1996), Spain (Cerrillo *et al.*, 1996), New Caledonia (Bailly *et al.*, 1996), Mexico (Rosiles *et al.*, 1998) and Iran (Raooofi *et al.*, 2003). Additionally cases of porcine pulmonary edema (PPE) have been associated with fumonisin-contaminated feeds in the United States (Harrison *et al.*, 1990; Osweiler *et al.*, 1992), Brazil (Sydenham *et al.*, 1992), Hungary (Fazekas *et al.*, 1998) and Thailand (Patchimasiri *et al.*, 1998).

Commercial corn-based human feedstuffs from retail outlets in several countries frequently contain fumonisins (Sydenham *et al.*, 1991; Pittet *et al.*, 1992; Stack and Eppley, 1992). Corn meal and corn grits appear to be the biggest problems, as up to 2.98 µg FB<sub>1</sub>/g and 0.92 µg FB<sub>2</sub>/g have been found in corn meal and up to 2.55 µg FB<sub>1</sub>/g and 1.07 µg FB<sub>2</sub>/g in corn grits (Sydenham *et al.*, 1991). Only very low incidences and levels of FB<sub>1</sub> have been recorded for corn flakes breakfast cereal in Switzerland, the United States and South Africa (Sydenham *et al.*, 1991; Pittet *et al.*, 1992; Stack *et al.*, 1992). A survey in Maryland showed that nearly 100% of corn-based food products (including corn muffin mixes, corn chips, corn tortillas, cornflakes breakfast cereal, corn starch, infant mixed cereal and corn grits) purchased in grocery stores contained some level of fumonisins with concentrations ranging up to 7.5 µg/g (Castelo *et al.*, 1998). Corn-based food products purchased in Arizona and Nebraska were also frequently (>85%) contaminated with fumonisins with concentrations up to 5.2 µg/g being reported (Castelo *et al.*, 1998).

## PHARMCOKINETICS/TOXICOKINETICS

The pharmacokinetics of fumonisin B<sub>1</sub> have been examined in several species including rats, pigs, cattle, laying hens and primates (Prelusky *et al.*, 1994, 1995; Vudathala *et al.*, 1994; Shephard *et al.*, 1995; Richard *et al.*, 1996; Martinez-Larranaga *et al.*, 1999). In general, fumonisin is rapidly absorbed following intravenous or intraperitoneal administration and is eliminated in both the feces and urine. Levels are undetectable by 24 hours after dosing in virtually all species and significant concentrations (residues) have not been found in muscle, milk or eggs. Following oral dosing, very little fumonisin B<sub>1</sub> is typically found in the serum of animals indicating low bioavailability.

More specifically, the toxicokinetics of radiolabeled fumonisin B<sub>1</sub> were examined after intragastric (0.5 mg fumonisin B<sub>1</sub>/kg) or intravenous (0.4 mg fumonisin B<sub>1</sub>/kg) administration to bile-cannulated and non-cannulated pigs (Prelusky *et al.*, 1994). Fumonisin-derived radioactivity was not detected in the plasma of pigs

dosed intravenously after 180 minutes in the non-cannulated group, or after 90 minutes in the cannulated group. Urinary excretion began within 3 hours of administration and virtually ended after 8 hours, accounting for only a small amount of administered toxin. Fecal excretion of fumonisin persisted for 48 hours. The excretion in the intravenously dosed group occurred primarily via the bile, with biliary excretion greatest during the first 4 hours, but persisting for 24–36 hours.

Plasma radioactivity in intragastrically dosed pigs was first detected 30–45 minutes after dosing, with maximal activity present between 60 and 90 minutes. As reflected in plasma and elimination data, systemic bioavailability of the dose ranged from 3 to 6%. Excretion of the fumonisin occurred primarily via feces, with only trace amounts excreted via urine or bile.

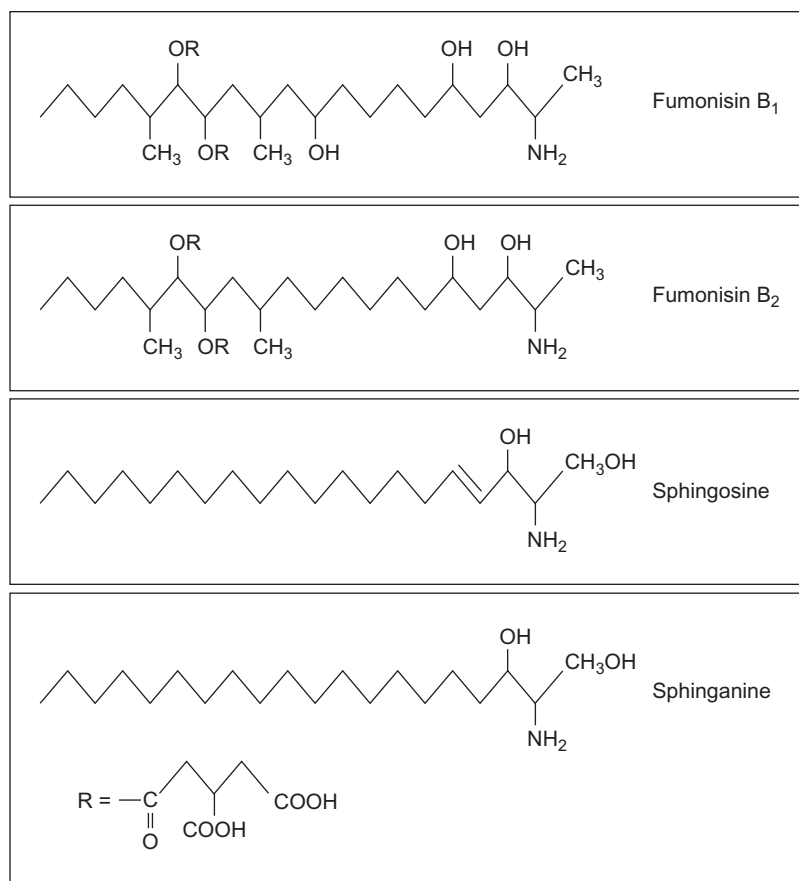
At 72 hours after administration, tissue radioactivity was highest in the liver, kidney and large intestine in all groups. Intragastrically dosed groups had 10–20-fold lower tissue concentrations than did intravenously dosed groups, and only intravenously dosed groups had measurable radioactivity in brain, lung and adrenal. Thus, it seems that liver and kidney are the primary organs of fumonisin metabolism and excretion in the pig, and that enterohepatic circulation prolongs the persistence of fumonisin in the body. The toxicokinetics of fumonisin B<sub>1</sub> in horses has not been evaluated.

When pigs were fed fumonisin at daily concentrations between 50 and 500 µg of fumonisin B<sub>1</sub> per kg of body weight for the last 5 months before slaughter, no muscle or kidney residues were detected (Liguoro *et al.*, 2004). Fumonisin B<sub>1</sub> was not detected in the eggs from laying hens following either intravenous or oral administration (Vudathala *et al.*, 1994). Although negligible concentrations have been shown to cross the mammary barrier (Spotti *et al.*, 2001), the toxin was not detected in milk from cattle that consumed a diet containing fumonisins (Richard *et al.*, 1996). Therefore, it appears that fumonisin residues in meat, milk or eggs does not represent a hazard or food safety concern for humans consuming these products.

## MECHANISM OF ACTION

### Sphingolipid alterations

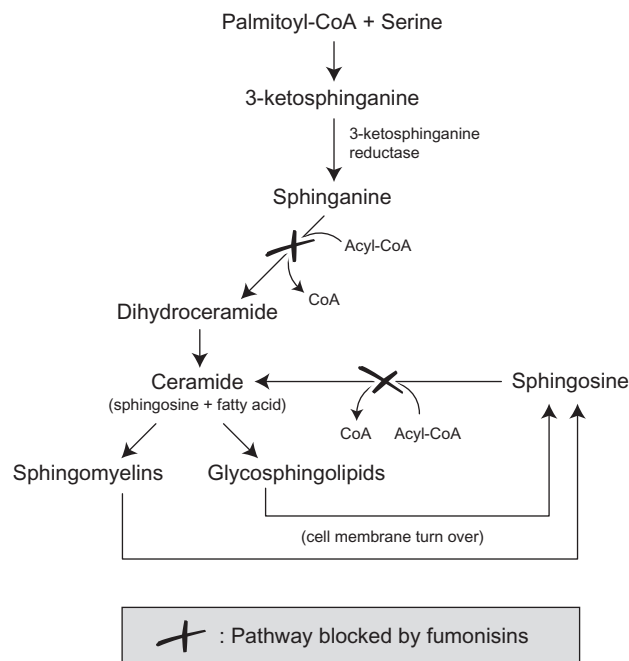
Fumonisin is structurally related to sphingosine, the major long chain base backbone of cellular sphingolipids (Figure 90.1). They are competitive inhibitors of sphingosine and sphingosine N-acyltransferase (also known as ceramide synthase), key enzymes in the *de novo* sphingolipid biosynthetic pathway (Figure 90.2). These N-acyltransferase enzymes are responsible for catalyzing



**FIGURE 90.1** The structure of fumonisin B<sub>1</sub>, fumonisin B<sub>2</sub>, sphingosine and sphinganine. (Modified from Diaz and Boermans, 1994, reprinted with permission from Smith and Constable, 2004.)

the acylation of sphinganine and the reutilization of sphingosine derived from sphingolipid turnover. This inhibition by fumonisin has been characterized *in vitro* using liver and brain microsomes, as well as in intact mammalian cells in culture (hepatocytes, neurons, renal cells and macrophages) (Merrill *et al.*, 1995). Fumonisin B<sub>1</sub> blocks the incorporation of radiolabeled serine into the sphingoid base backbone of ceramides and complex sphingolipids and prevents the conversion of sphinganine to sphingosine via addition of the 4,5 *trans* double bond, which occurs after acylation of sphinganine. Fumonisin also blocks reacylation of sphingoid bases (primarily sphingosine) released by hydrolysis of more complex sphingolipids (Merrill *et al.*, 1995).

Sphingolipids are located in cellular membranes, lipoproteins (especially low-density lipoproteins) and other lipid-rich structures. Complex sphingolipids are critical for the maintenance of membrane structure, particularly microdomains such as caveolae. They also serve as binding sites for extracellular matrix proteins as well as for some microorganisms, microbial toxins and viruses, and regulate the behavior of growth factor receptors (Merrill



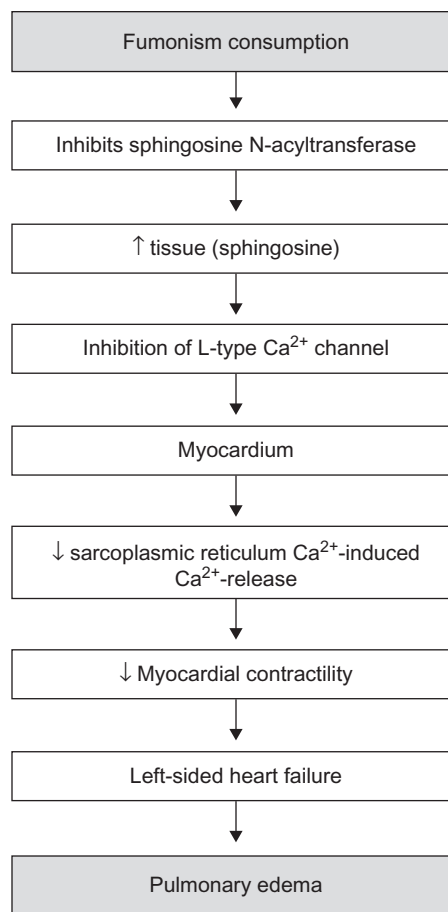
**FIGURE 90.2** The effects of fumonisin on the sphingolipid biosynthetic pathway. (Modified from Diaz and Boermans, 1994, reprinted with permission from Smith and Constable, 2004.)

and Sweeley, 1996). Complex sphingolipids function as precursors for second messengers that mediate cell responses to growth factors, cytokines (including tumor necrosis factor- $\alpha$ ), differentiation factors and 1,25-dihydroxy-vitamin D<sub>3</sub>. Therefore sphingolipids are involved in the regulation of cell growth, cell-to-cell communication, differentiation and neoplastic transformation (Hannun and Bell, 1989).

This enzyme inhibition by fumonisin produces a disruption of sphingolipid metabolism resulting in increased sphinganine and sphingosine along with a decrease in complex sphingolipids in the serum and tissues of animals (Wang *et al.*, 1991). These elevations in concentrations of sphinganine and sphingosine have also been observed *in vivo* in several species including pigs, horses and calves (Riley *et al.*, 1993; Goel *et al.*, 1996; Smith *et al.*, 1999, 2000; Mathur *et al.*, 2001). This disruption of sphingolipid metabolism is generally accepted as the probable mechanism of fumonisin toxicity; however, only in pigs has the pathophysiology been definitively determined.

Porcine pulmonary edema has been shown to be a direct result of acute left-sided heart failure related to an increase in plasma and myocardial sphinganine and sphingosine concentrations (Constable *et al.*, 2000; Smith *et al.*, 1999, 2000). Sphingosine is an important intracellular second messenger that inhibits L-type calcium channels in myocardial cells, thereby decreasing sarcoplasmic reticulum Ca<sup>2+</sup>-induced Ca<sup>2+</sup>-release and cardiac contractility (McDonough *et al.*, 1994; Webster *et al.*, 1994). As sphingosine concentrations begin to increase in pigs that consume fumonisin, myocardial calcium channels are blocked and contractility begins to decrease (Constable *et al.*, 2000; Smith *et al.*, 2000). Ultimately this decrease in cardiac contractility causes acute left ventricular failure and pulmonary edema (Figure 90.3).

The mechanism of equine leukoencephalomalacia may also be a direct result of fumonisin-induced increases in sphingosine concentrations. A recent study demonstrated that fumonisin administration induced cardiovascular dysfunction in horses (Smith *et al.*, 2002). This study reported an association between neurologic signs, increased serum and myocardial sphingosine concentrations, and cardiovascular depression in fumonisin-treated horses. At necropsy, horses with leukoencephalomalacia have histologic evidence of cerebral edema in the brain. Another study has reported that fumonisin-treated horses also have elevated protein, albumin and IgG levels in cerebrospinal fluid samples (Foreman *et al.*, 2004). Taken together, these findings indicate that fumonisin toxicity in horses is associated with the development of vasogenic cerebral edema as a direct result of increased blood-brain barrier permeability. Horses are a species that is dependent on autoregulation of cerebral blood flow when they lower their



**FIGURE 90.3** Mechanism of fumonisin-induced pulmonary edema in swine. Fumonisin inhibition results in increased tissue sphingosine and sphinganine concentrations. The increased sphingosine concentrations inhibit the L-type Ca<sup>2+</sup> channels of cardiac myocytes resulting in decreased myocardial contractility. This decrease in contractility results in acute left-sided heart failure and pulmonary edema.

head to graze. Because of gravitational forces, distal carotid artery pressure can increase tremendously when the animal bends to eat or drink. However, this rise in carotid pressure does not create a significant increase in cerebral blood flow due to the constriction and dilation of cerebral arterioles which maintain normal cerebral blood pressures (Faraci and Heistad, 1990). It has been shown that L-type calcium channels are the primary mediators of vascular tone in these cerebral arterioles (Michelakis *et al.*, 1994). Therefore, it has been hypothesized that fumonisin-induced increases in sphingosine concentrations inhibit the calcium channels in cerebral arterioles leading to the inability to maintain normal cerebral blood pressure and vasogenic cerebral edema. This hypothesis will require further research to be proven definitively.



## TOXICITY

Fumonisin has been shown to cause liver damage in multiple species including pigs, horses, cattle, rabbits and primates (Jaskiewicz *et al.*, 1987; Voss *et al.*, 1989; Haschek *et al.*, 1992; Osweiler *et al.*, 1993; Ross *et al.*, 1993; Gumprecht *et al.*, 1995) as well as species-specific target organ toxicity, such as lung in pigs (Haschek *et al.*, 1992), brain in horses (Ross *et al.*, 1993), kidney in rats, rabbits and sheep (Voss *et al.*, 1989; Edrington *et al.*, 1995; Gumprecht *et al.*, 1995) and esophagus in rats and pigs (Casteel *et al.*, 1993; Lim *et al.*, 1996). This chapter is focused on fumonisin toxicity in pigs and horses since they are the most common clinical poisonings dealt with in veterinary medicine; however, cattle and poultry are briefly discussed.

### Spontaneous and experimental fumonisin toxicosis in swine

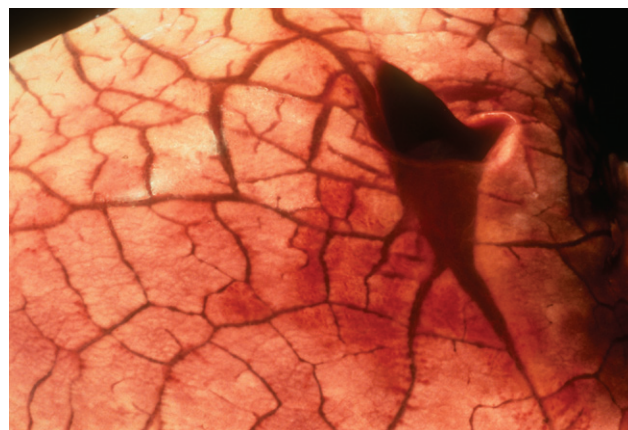
In early research prior to the initial isolation and characterization of fumonisins, *Fusarium verticillioides* culture material was reported toxic to swine (Kriek *et al.*, 1981). In that experiment, three pigs were fed *F. verticillioides* culture material grown on corn. Two of the three pigs fed the culture material in this study died within 5 days of pulmonary edema. The third pig was fed culture material for 89 days and was then killed following a period of feed refusal.

The 1989 corn crop in many Midwestern and southeastern parts of the United States was heavily infected with *F. verticillioides*, and contaminated screenings fed to animals led to fatal outbreaks of porcine pulmonary edema (Harrison *et al.*, 1990; Osweiler *et al.*, 1992). This syndrome was also reproduced experimentally with contaminated corn screenings and purified fumonisin B<sub>1</sub> (Harrison *et al.*, 1990; Osweiler *et al.*, 1992).

Lung and liver are the major target organs of fumonisin toxicosis in pigs; however, other organs have been reported to be affected. Pigs which ingest fumonisin at concentrations high enough to cause pulmonary edema usually die after about 4 days in field cases (Osweiler *et al.*, 1992) and after 3–6 days of fumonisin exposure experimentally (Haschek *et al.*, 1992; Motelin *et al.*, 1994; Gumprecht *et al.*, 1998). Pigs which survive chronic exposure to high doses of fumonisin without developing pulmonary edema typically demonstrate hepatic disease with anorexia, weight loss and generalized icterus (Osweiler *et al.*, 1992; Colvin *et al.*, 1993). Hepatic toxicity occurs at doses significantly lower than those necessary to cause pulmonary edema (Colvin *et al.*, 1993; Motelin *et al.*, 1994).

### Fumonisin in swine – pulmonary effects

Pulmonary edema (Figure 90.4) has been reported in pigs fed naturally contaminated fumonisin-containing



**FIGURE 90.4** Lung from a pig fed fumonisin-containing culture material at a dose of 20 mg fumonisin B<sub>1</sub> per kg of body weight for 4 days. Pulmonary edema is characterized by severe widening of the interlobular septa. (Reprinted with permission from Smith and Constable, 2004.)

food, fumonisin-containing culture material or following IV administration of fumonisin (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Motelin *et al.*, 1994). Reported concentrations of fumonisin required to produce pulmonary edema have been variable, presumably due to variability in susceptibility among exposed animals (Table 90.1). However, other constituents in the diet and analytical detection related to the ability to extract fumonisin from different matrices could account for some of the variability. In addition, some reports have documented only the concentration of fumonisin B<sub>1</sub> associated with the development of pulmonary edema, while others have reported both fumonisin B<sub>1</sub> and fumonisin B<sub>2</sub>. Fumonisin B<sub>2</sub> usually occurs at about 30% of fumonisin B<sub>1</sub> in naturally contaminated corn, and is generally considered to be equitoxic to fumonisin B<sub>1</sub> (Ross *et al.*, 1994). Reported doses that induced pulmonary edema in swine include 100 ppm of fumonisin B<sub>1</sub> and fumonisin B<sub>2</sub> in naturally contaminated corn (Motelin *et al.*, 1994), 16 mg fumonisin B<sub>1</sub>/kg/day as fumonisin-containing culture material (Colvin *et al.*, 1993) and 20 mg fumonisin B<sub>1</sub>/kg/day as culture material (Gumprecht *et al.*, 1998). Fumonisin-induced pulmonary edema has also been reported with naturally contaminated corn (330 mg of fumonisin B<sub>1</sub> per kg of feed) in Hungary (Fazekas *et al.*, 1998), Brazil (Sydenham *et al.*, 1992) and Thailand (Patchimasiri *et al.*, 1998).

A more recent study has suggested that even lower concentrations of fumonisins may be able to induce pulmonary edema in swine (Zomborszky *et al.*, 2000). Fumonisin B<sub>1</sub> was added to the feed of weaned pigs at doses of 0, 10, 20 and 40 ppm for 4 weeks as fumonisin-containing culture material (five pigs per group). Computed tomography of the lungs and magnetic

TABLE 90.1 Effects of fumonisin in pigs

Number of animals	Dose and route	Duration	Toxic effects	Reference
<b>Experimental studies using purified fumonisin</b>				
4 pigs	0.174 to 0.4 mg FB <sub>1</sub> per kg of BW/day, IV	4 to 8 days	2 high dose pigs developed pulmonary edema	Harrison <i>et al.</i> (1990)
2 pigs	0.88 to 1.15 mg FB <sub>1</sub> per kg of BW/day, IV	1 pig for 4 days (0.88 mg/kg/day) – 2nd pig for 9 days (1.15 mg/kg/day)	Mild interstitial pulmonary edema histologically in one pig; hepatic lesions; pancreatic lesions	Haschek <i>et al.</i> (1992)
3 pigs	4.5 to 6.6 mg FB <sub>1</sub> per kg of BW/day in feed	5 to 15 days	2 of the 3 pigs developed severe pulmonary edema; hepatic lesions and mild pancreatic lesions noted	Haschek <i>et al.</i> (1992)
2 pigs	0.4 mg FB <sub>1</sub> per kg of BW/day, IV	12 to 14 days	Elevated AST, GGT, bilirubin; liver lesions	Osweiler <i>et al.</i> (1992)
8 pigs	10 ppm FB <sub>1</sub> added to the diet – fed ad libitum	8 weeks	Decreased weight gain	Rooter <i>et al.</i> (1996)
6 pigs	1 mg FB <sub>1</sub> per kg of BW/day, IV	4 days	Elevated cholesterol Decrease in left ventricular contractility and mild pulmonary edema	Smith <i>et al.</i> (2000)
<b>Experimental studies using fumonisin-containing culture material</b>				
6 pigs	Feeds containing 100 and 190 ppm FB <sub>1</sub> – fed ad libitum	100 ppm diet fed ad libitum for 7 days followed by 190 ppm diet for 83 days	Elevated AST, ALP, GGT; nodular hyperplasia of the liver; histologic esophageal lesions	Casteel <i>et al.</i> (1993)
4 pigs	Feed containing 200 ppm of FB <sub>1</sub> – fed ad libitum	Up to 43 days	Elevated bilirubin, AST, cholesterol; hepatic lesions; decreased weight gain	Colvin <i>et al.</i> (1993)
7 pigs	16 to 64 mg FB <sub>1</sub> per kg of BW/day as oral gavage	3 to 5 days	All pigs developed pulmonary edema within 5 days	Colvin <i>et al.</i> (1993)
3 pigs	4 to 16 mg FB <sub>1</sub> per kg of BW/day as oral gavage	Up to 45 days	Severe hepatic disease; icterus; elevated liver enzymes; no pulmonary edema	Colvin <i>et al.</i> (1993)
11 pigs	Feeds containing 100, 160 and 190 ppm FB <sub>1</sub> – fed ad libitum	6 pigs were fed 100 ppm for 10 days then 190 ppm for up to 83 days; 5 pigs were fed 100 ppm for 5 days and then 160 ppm for up to 205 days	Nodular hyperplasia of the liver; elevated AST, ALP, GGT, bilirubin Right ventricular hypertrophy; medial hypertrophy of the small pulmonary arteries	Casteel <i>et al.</i> (1994)
6 pigs	Feed containing 47 ppm FB <sub>1</sub> – fed ad libitum	28 days	Decreased feed consumption; elevated AST, GGT, ALP and creatinine; hepatic and renal lesions; medial hypertrophy of the pulmonary arteries	Harvey <i>et al.</i> (1996)
10 pigs	20 mg of FB <sub>1</sub> per kg of BW/day, in feed	7 days	Pulmonary edema and cardiovascular abnormalities	Smith <i>et al.</i> (1996a); Smith <i>et al.</i> (1996b)
2 pigs	14.5 and 16 mg of FB <sub>1</sub> per kg of BW/day, in feed	4 days	Severe pulmonary edema; renal lesions	Fazekas <i>et al.</i> (1997)
24 pigs	20 mg of FB <sub>1</sub> per kg of BW/day, in feed	Up to 5 days – some pigs were euthanized each day of the study	All 12 pigs euthanized on days 0, 1 and 2 had no lesions 2/5 day 3 pigs and all of the day 4 and 5 pigs had pulmonary edema Elevations in liver enzymes and hepatic lesions seen on day 2 – bile acids first liver parameter to increase	Gumprecht <i>et al.</i> (1998)
6 pigs	20 mg of FB <sub>1</sub> per kg of BW/day, in feed	5–6 days – pigs were euthanized as they developed pulmonary edema	Increased pulmonary artery pressure, pulmonary artery wedge pressure and decreased cardiac output, heart rate and mean arterial pressure	Smith <i>et al.</i> (1999)
5 pigs	Feed containing 40 ppm FB <sub>1</sub> – fed ad libitum	4 weeks	No clinical signs – gross pulmonary edema evident at necropsy	Zomborszky <i>et al.</i> (2000)
5 pigs	Feed containing 20 ppm FB <sub>1</sub> – fed ad libitum	4 weeks	2 of the 5 pigs had gross pulmonary edema – 2 additional pigs had mild evidence of edema present histologically	Zomborszky <i>et al.</i> (2000)

(Continued)

TABLE 90.1 (Continued)

Number of animals	Dose and route	Duration	Toxic effects	Reference
4 pigs	Feed containing 20 ppm FB <sub>1</sub> – fed ad libitum	28 days	3 of the 4 pigs had mild pulmonary edema evident histologically	Zomborszky <i>et al.</i> (2000)
7 pigs	20 mg of FB <sub>1</sub> per kg of BW/day, in feed	3 days	Decreased left ventricular contractility; heart rate, cardiac output, and mechanical efficiency of the left ventricle	Constable <i>et al.</i> (2000)
<b>Experimental studies using naturally contaminated corn screenings</b>				
6 pigs	105 to 155 ppm FB <sub>1</sub> in corn screenings – fed ad libitum	Up to 28 days	3 of 6 pigs developed pulmonary edema; liver and pancreatic lesions	Harrison <i>et al.</i> (1990)
8 pigs	92 ppm FB <sub>1</sub> in corn screenings – fed ad libitum	Up to 21 days	6 of 8 pigs developed pulmonary edema; the remaining 2 pigs were icteric with increased liver enzymes	Osweiler <i>et al.</i> (1992)
5 pigs	175 ppm (FB <sub>1</sub> + FB <sub>2</sub> ) as corn screenings mixed in a complete ration	Up to 14 days	3 of 5 pigs developed pulmonary edema; hepatotoxicity; decreased weight gain	Motelin <i>et al.</i> (1994)
5 pigs	101 ppm (FB <sub>1</sub> + FB <sub>2</sub> ) as corn screenings mixed in a complete ration	Up to 14 days	Elevated GGT, ALT, AST, ALP and bilirubin; liver lesions; decreased weight gain	Motelin <i>et al.</i> (1994)
5 pigs	39 ppm (FB <sub>1</sub> + FB <sub>2</sub> ) as corn screenings mixed in a complete ration	14 days	Histologic liver lesions	Motelin <i>et al.</i> (1994)
5 pigs	39 ppm (FB <sub>1</sub> + FB <sub>2</sub> ) as corn screenings mixed in a complete ration	14 days	Histologic liver lesions	Motelin <i>et al.</i> (1994)
<b>Reported fumonisin concentrations from naturally occurring outbreaks</b>				
34 pigs from 2 farms	105 to 155 ppm FB <sub>1</sub>	Unknown	Lethal pulmonary edema	Harrison <i>et al.</i> (1990)
16 pigs from 9 farms	All feed samples associated with pulmonary edema contained $\geq 20$ ppm FB <sub>1</sub> to a maximum of 330 ppm FB <sub>1</sub>	Unknown	Lethal pulmonary edema	Osweiler <i>et al.</i> (1992)

resonance imaging of the brains were performed prior to the study and at 2 and 4 weeks of fumonisin feeding. Histopathology was also done at the time of necropsy (4 weeks). The results of this study showed that all five pigs fed fumonisin B<sub>1</sub> at 40 ppm developed “severe” pulmonary edema as assessed by computed tomography and histopathology. Two of the five pigs fed fumonisin B<sub>1</sub> at 20 ppm had “severe” pulmonary edema while two other pigs in the group had “mild” edema. In the 10 ppm group, three of the five pigs were reported to have “mild” pulmonary edema. Magnetic resonance studies of the brain were not able to identify any significant changes during the course of the study in any group.

Clinical signs associated with the development of pulmonary edema consistently begin 3–6 days after initiation of exposure to a high concentration of fumonisins. These include dyspnea and open-mouthed breathing, increased respiratory rate, cyanosis of skin and mucous membranes, inactivity and sudden death (Osweiler *et al.*, 1992). Pigs usually die within a few hours after the onset of definitive respiratory distress. Histologically, pulmonary edema is present by day 3 of fumonisin exposure

(Gumprecht *et al.*, 1998) and is characterized by interstitial edema around airways and vessels, in interlobular and subpleural connective tissues, and in alveolar interstitium (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Gumprecht *et al.*, 1998). Lymphatics are dilated and alveolar edema is often present. Fluid is also present within the thoracic cavity.

In ultrastructural studies using immersion fixed lungs, the endothelium was found to be swollen, vacuolated and sometimes missing in pigs with pulmonary edema (Haschek *et al.*, 1992). Additional studies using intravascularly perfused lungs (to allow better examination of the vascular system) demonstrated accumulations of fragmented membranous material in the cytocavitary region of endothelial cells (Gumprecht *et al.*, 1998).

### Fumonisin in swine – hepatic effects

Hepatic changes in pigs exposed to fumonisins include elevation of liver-associated enzyme activities, altered clinical chemistries, changes in sphingolipid parameters

and morphological alterations. In pigs, hepatic toxicity occurs prior to the development of pulmonary edema, and alterations are time and dose dependent (Motelin *et al.*, 1994). Increased activities of serum enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (GGT) and concentrations of total bilirubin, bile acids and cholesterol have been reported as early as 1 day after the initiation of fumonisin exposure (Harrison *et al.*, 1990; Haschek *et al.*, 1992; Osweiler *et al.*, 1992; Colvin *et al.*, 1993; Motelin *et al.*, 1994; Gumprecht *et al.*, 1998). These alterations reflect hepatocyte damage as well as altered hepatic function.

Morphologic alterations are dose related and progressive with continued ingestion of fumonisins. Following short-term exposure, changes include hepatic cord disorganization, cytoplasmic vacuolation, apoptosis, scattered necrosis and increased cell proliferation (Harrison *et al.*, 1990; Osweiler *et al.*, 1992; Motelin *et al.*, 1994; Gumprecht *et al.*, 1998). Histologic alterations were observed as early as 2 days after the initiation of treatment with a lethal dose (Gumprecht *et al.*, 1998), and at a concentration as low as 23 ppm when fed for 14 days (Motelin *et al.*, 1994). Long-term fumonisin exposure can result in fibrosis or development of hyperplastic nodules in the liver (Harrison *et al.*, 1990; Casteel *et al.*, 1993). Ultrastructurally, large accumulations of proteinaceous and membranous material were observed in the Space of Disse in pigs that developed fumonisin-induced pulmonary edema (Haschek *et al.*, 1992). Hepatocytes lost microvilli from their sinusoidal face while numerous Kupffer cells contained multilamellar bodies.

### Fumonisin in swine – cardiovascular effects

Fumonisin have been shown to decrease left ventricular contractility, heart rate, cardiac output, mean arterial pressure, arterial and mixed venous blood O<sub>2</sub> tensions, and systemic oxygen delivery, while increasing mean pulmonary artery pressure, oxygen consumption and oxygen extraction ratio in swine (Constable *et al.*, 2000; Smith *et al.*, 1996a,b, 1999, 2000). The decrease in cardiac contractility leads to acute left ventricular failure and pulmonary edema in pigs exposed to high concentrations of fumonisin in feed. Chronic exposure to lower levels of fumonisin leads to the development of right ventricular hypertrophy and medial hypertrophy of the small pulmonary arteries in pigs, likely a result of pulmonary hypertension (Casteel *et al.*, 1994).

### Fumonisin in swine – immunologic effects

Fumonisin have also been shown to predispose pigs to respiratory disease. In one case-control study, swine

farms with  $\geq 20$  ppm of fumonisin in the feed were at significantly greater risk for pneumonia as compared to farms with low fumonisin concentrations (Bane *et al.*, 1992). As the concentration of fumonisin in the feed increased, the risk of respiratory disease continued to increase. Later it was shown that exposure to fumonisins depressed pulmonary intravascular macrophage function, and pigs exposed to this toxin had decreased pulmonary clearance of blood-borne particulates and bacteria when compared to control animals (Smith *et al.*, 1996c). In a more recent study, exposure to fumonisin exacerbated respiratory disease in a *Pasteurella multocida* challenge model (Halloy *et al.*, 2005). Pigs that were fed 0.5 mg of FB<sub>1</sub> per kg of body weight/day for 7 days had delayed growth, increased coughing and more severe lung lesions than control pigs. Therefore, at levels well below those needed to cause hepatic lesions or pulmonary edema, fumonisins are likely to reduce growth rates and increase disease in pigs.

### Fumonisin toxicosis in horses – historical

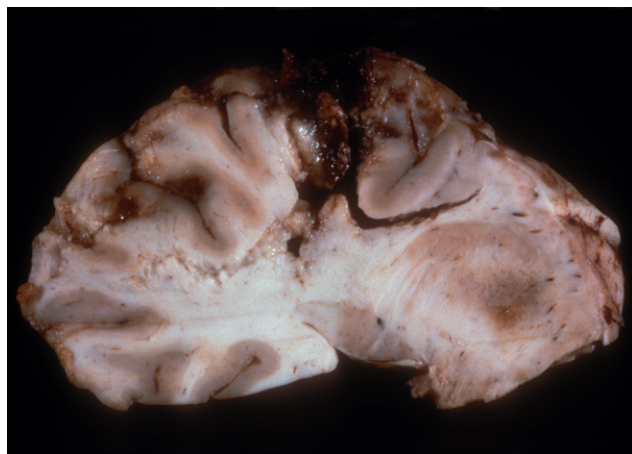
Several outbreaks of a neurologic disease in horses occurred in the United States in the early 1900s with thousands of deaths reported in several states. The earliest citation of neurologic deaths associated with the feeding of contaminated corn was from Maryland (MacCallum and Buckley, 1902). The condition was commonly referred to as “cerebrospinal meningitis” and presented with fairly characteristic signs. The duration of disease varied from a few hours to a week, and the brains from affected horses had “softened” areas in the cerebrum involving only the white matter. Additional outbreaks were subsequently reported from Kansas, Iowa, Mississippi and North Carolina. A similar disease, described as “epizootic cerebritis,” had been encountered in 1891; however, it is not known whether this was associated with corn (Butler, 1902). When feed from an outbreak of “leucoencephalitis” in Kansas was fed to a horse, it died after developing neurologic signs (Butler, 1902). At necropsy, the left cerebral hemisphere was “soft to the touch, and when cut through, the white matter was broken down extensively, nearly the entire hemisphere being involved.” Several attempts were made to identify an infectious agent in the brain of affected horses and all were negative (MacCallum and Buckley, 1902). The authors concluded that a toxic etiology was likely.

In central Illinois, more than 5000 horses died during the winter of 1934 to 1935 from a syndrome referred to as “cornstalk disease” (Graham, 1935). Brain tissue suspensions and filtrates were inoculated into laboratory animals; however, no infectious etiology could be identified. Graham then placed eight horses into a field containing cornstalks in Rantoul, Illinois. Two of these animals died



23 and 26 days after being placed in the field (Graham, 1936). These outbreaks were similar to those reported in 1893 and 1914 which had occurred following a summer drought (Graham, 1936). Neurologic deaths with similar lesions were also reported in Iowa during the winter of 1914 and in the spring of 1935 (Schwarte *et al.*, 1937). Histologic examination of the brain revealed no evidence of infectious agents. This “syndrome” was then subsequently reproduced by feeding moldy corn and corn fodder to five horses (Schwarte *et al.*, 1937). A similar disease syndrome was identified and confirmed by feeding trials in Egypt (Badiali *et al.*, 1968; Wilson and Maronpot, 1971) and in South Africa (Marasas *et al.*, 1976). At necropsy, these studies were able to consistently demonstrate swelling of the cerebral hemispheres and flattening of the overlying gyri. On coronal sections, there were cavities of varying sizes with liquefactive necrosis of subcortical white matter in one or both cerebral hemispheres (Figure 90.5). There were also scattered multifocal hemorrhages in the surrounding white matter (Marasas *et al.*, 1976; Haliburton *et al.*, 1979). Based on these findings, Marasas *et al.* (1976) coined the term “equine leukoencephalomalacia” as a distinct clinical and morphologic syndrome in horses associated with the feeding of corn.

*Fusarium verticillioides* was later isolated from corn collected from field outbreaks in Egypt, and leukoencephalomalacia was subsequently reproduced in donkeys fed corn inoculated with the fungus (Wilson and Maronpot, 1971). In South Africa, however, samples of corn inoculated with *F. verticillioides* produced liver damage and icterus in several horses and donkeys, but not brain lesions (Kellerman *et al.*, 1972). Marasas *et al.* (1976) then produced a batch of culture material using a strain of *F. verticillioides* from an outbreak of leukoencephalomalacia



**FIGURE 90.5** A cross-section of a cerebral hemisphere from a horse demonstrating liquefactive necrosis of the white matter typical of equine leukoencephalomalacia. (Reprinted with permission from Smith and Constable, 2004.)

to produce liver damage and neurologic disease in a horse. It was then concluded that both hepatic disease and equine leukoencephalomalacia (ELEM) were manifestations of the same toxicosis, with different clinical syndromes occurring depending on toxin dose and length of exposure. Following the isolation and purification of fumonisin B<sub>1</sub> in the late 1980s, ELEM was experimentally induced by the administration of purified toxin (Marasas *et al.*, 1988; Kellerman *et al.*, 1990).

### Spontaneous and experimental fumonisin toxicosis in horses

Since the discovery of fumonisin B<sub>1</sub> as the causative agent of ELEM, many more disease outbreaks associated with the feeding of corn have been reported (Wilson *et al.*, 1990a; Ross *et al.*, 1991, 1993; Uhlinger, 1991; Binkerd *et al.*, 1993; Christley *et al.*, 1993; Wilkins *et al.*, 1994; Bailly *et al.*, 1996; Bela and Endre, 1996; Cerrillo *et al.*, 1996; Rosiles *et al.*, 1998). Purified fumonisin B<sub>1</sub> has induced equine leukoencephalomalacia (ELEM) when administered orally (Kellerman *et al.*, 1990) and intravenously (Marasas *et al.*, 1988; Laurent *et al.*, 1989; Foreman *et al.*, 2004). Purified fumonisin B<sub>2</sub> has also induced ELEM when given orally (Ross *et al.*, 1994). Fumonisins B<sub>3</sub> is suspected to be much less toxic than either B<sub>1</sub> or B<sub>2</sub>. Fumonisin B<sub>1</sub> is considered to be the primary cause of ELEM, however, as fumonisin B<sub>2</sub> is usually present in concentrations that are 20–40% of fumonisin B<sub>1</sub> (Ross *et al.*, 1991). Although ELEM has occurred in horses eating commercial feedstuffs (Wilson *et al.*, 1990b; Ross *et al.*, 1991), the feeding of corn screenings has been more frequently associated with ELEM, because fumonisin concentrations are much higher in screenings than in whole kernels of corn (Binkerd *et al.*, 1993). Fumonisin B<sub>1</sub> also appears to survive the pelleting process for equine feeds (Ross *et al.*, 1991).

Leukoencephalomalacia has been reproduced with intravenous administration of fumonisin B<sub>1</sub> in three separate studies (Marasas *et al.*, 1988; Laurent *et al.*, 1989; Foreman *et al.*, 2004). Marasas *et al.* (1988) administered 0.125 mg fumonisin B<sub>1</sub>/kg of body weight, IV, q24h which produced ELEM in 9 days. Laurent *et al.* (1989) administered 0.1 mg fumonisin B<sub>1</sub>/kg of body weight, IV, q24h for 16 days followed by 0.2 mg/kg/day for 2 additional days. Leukoencephalomalacia was induced in 18 days. Foreman *et al.* (2004) administered 0.05, 0.1 or 0.2 mg fumonisin B<sub>1</sub>/kg of body weight, IV, q24h to 10 horses and all developed neurologic signs and were euthanized between days 4 and 12 of the study. In contrast, horses dosed with 0.01 mg fumonisin B<sub>1</sub>/kg of body weight for 28 days in this study did not develop neurologic signs. Purified fumonisin B<sub>1</sub> has also been administered orally in other studies (1.25 or 2.5 mg

fumonisin B<sub>1</sub>/kg of body weight, PO, q24h), producing mild edema of the brain stem and hepatic disease in 11–12 days in two horses. In a subsequent study, animals were fed 0.6 to 4.0 mg fumonisin B<sub>1</sub>/kg of body weight, PO, q24h for 33 or 35 days, producing hepatotoxicity and neurologic signs starting on days 22 and 24 in two weanling horses (Kellerman *et al.*, 1990).

Doses of fumonisin reported from naturally occurring cases of fumonisin have varied (Table 90.2). One field report calculated that the ingestion of 0.6 to 2.1 mg fumonisin B<sub>1</sub>/kg of body weight would induce ELEM in 24–28 days (Wilson *et al.*, 1990b). Another study found that leukoencephalomalacia was associated with ingestion of feed containing fumonisin B<sub>1</sub> concentrations greater than 10 ppm, and concluded that feed with fumonisin B<sub>1</sub> concentrations greater than 10 ppm was not safe to be fed to horses (Ross *et al.*, 1991).

### Neurologic and hepatic effects in horses

Several reports have considered ELEM and hepatotoxicity to be two separate syndromes associated with fumonisin toxicity in horses, with the terms “classic neurotoxic syndrome” and “hepatic syndrome” being used (McCue, 1989). However, it appears more likely these are not true “distinct” syndromes but are related to the concentration of fumonisin in the feed, the duration of toxin consumption and the tolerance of the individual horse to fumonisin. In some outbreaks, horses have died from ELEM while other horses have died from hepatotoxicity, and occasionally individual horses exhibiting both neurologic and hepatic signs have been described. Reported clinical signs associated with hepatic disease include icterus, mucous membrane petechiae and swelling of the lips or muzzle (Uhlinger, 1991; Ross *et al.*, 1993).

Ross *et al.* (1993) described an experimental study where one horse died acutely with “mild encephalopathy and hepatic necrosis” after 9 days of fumonisin exposure whereas two other horses died after 75 and 78 days of ELEM. The horse that died on day 9 showed neurologic signs prior to death (“visual impairment, mild ataxia and slight head tremors”) and had histologic evidence of leukoencephalomalacia at necropsy; however, his death was primarily attributed to hepatotoxicity. This study led to a common generalization that high doses of fumonisin were likely to induce hepatotoxicity, whereas lower doses of toxin over a longer period of time were necessary to induce ELEM (McCue, 1989; Plumlee and Galey, 1994). However, in other experimental studies, intravenous administration of fumonisin B<sub>1</sub> induced ELEM in 9 days (Marasas *et al.*, 1988) and 18 days (Laurent *et al.*, 1989). In a large study with varying doses of fumonisin, horses treated with higher doses developed leukoencephalomalacia (in 5–8 days), whereas

horses that received lower concentrations developed primarily hepatic lesions without any evidence of neurotoxicity (Foreman *et al.*, 2004). Therefore it can be concluded that ELEM results from an acute exposure to feed containing high concentrations of fumonisin B<sub>1</sub>, while hepatotoxicity occurs with chronic ingestion of lower levels.

Serum biochemical changes associated with fumonisin toxicity in horses have been predominantly related to hepatotoxicity: increased AST (Wang *et al.*, 1992); increased AST and GGT (Laurent *et al.*, 1989; Kellerman *et al.*, 1990); increased GGT and SDH (Schumacher *et al.*, 1995); increased AST, GGT and ALP (Ross *et al.*, 1993); increased AST, GGT, ALP, total bilirubin and bile acids (Wilson *et al.*, 1992); and “elevated liver enzymes” (Ross *et al.*, 1994).

The neurologic signs are usually summarized as sudden onset of one or more of the following: frenzy, aimless circling, head pressing, paresis, ataxia, blindness, depression and hyperexcitability (Ross *et al.*, 1991; Wilson *et al.*, 1992). Other reports have stated that “the disease started with lack of appetite, followed by the disturbance of swallowing and chewing indicating the paralysis of cephalic and pharyngeal muscles. Paralysis of cephalic and cervical muscles spread to the muscles of the extremities and trunk. The animals moved with difficulties, and tottering and ataxia developed. Signs of “blindness” developed in one animal. At the final stage of disease, the affected animals “laid down and died” (Bela and Endre, 1996). In a comprehensive study, early neurologic signs included mild proprioceptive abnormalities, including hindlimb ataxia, delayed forelimb placing reactions and decreased tongue tone and movement (Foreman *et al.*, 2004). These signs progressed over 12 to 48 hours to become more readily apparent. Hindlimb and trunk ataxia in particular became more apparent with time. A variety of behavioral changes were observed including depression, hyperesthesia and intermittent dementia. All horses had intact menace and pupillary light responses at the time of death.

Cerebrospinal fluid findings from horses with ELEM include elevations in protein concentration, albumin and IgG concentrations and increased albumin quotients (Foreman *et al.*, 2004). Cerebrospinal fluid, red blood cell, leukocyte and glucose concentrations along with creatine kinase activity are not altered in horses with neurologic disease. Along with the histopathologic findings, these cerebrospinal fluid changes indicate the presence of a vasogenic cerebral edema in horses with leukoencephalomalacia.

### Fumonisin toxicity in cattle

Adult beef cattle appear relatively resistant to fumonisin. Feeder calves fed a diet containing fumonisin concentrations up to 148 ppm for 31 days had only mild

TABLE 90.2 Effects of fumonisin in horses

Number of animals	Dose and route	Duration	Toxic effects	Reference
<b>Experimental studies using purified fumonisin</b>				
1 horse	0.125 mg/kg/day, IV	7 treatments over 9 days	Leukoencephalomalacia Elevated AST, GGT	Marasas <i>et al.</i> (1988)
1 horse	2.5 mg/kg/day, PO (by gavage)	6 doses over 11 days	Severe hepatosis Elevated AST, GGT	Marasas <i>et al.</i> (1988)
1 horse	1.25 mg/kg/day, PO (by gavage)	6 doses over 11 days	Mild hepatosis Elevated AST, GGT	Marasas <i>et al.</i> (1988)
1 horse	0.1 mg/kg/day, IV and 0.2 mg/kg/day, IV	0.1 mg/kg dose given for 16 days then 0.2 mg/kg dose given for 2 additional days	Leukoencephalomalacia	Laurent <i>et al.</i> (1989)
1 horse	1.25 mg/kg to 4.0 mg/kg, PO	20 doses given over 35 days	Leukoencephalomalacia Elevated AST	Kellerman <i>et al.</i> (1990)
1 horse	1.0 mg/kg to 4.0 mg/kg, PO	20 doses given over 33 days	Leukoencephalomalacia Elevated AST, GGT	Kellerman <i>et al.</i> (1990)
13 horses	0.01 to 0.20 mg/kg, IV	Up to 10 days	Leukoencephalomalacia at 0.10 and 0.20 mg/kg – hepatic toxicity only at lower doses	Smith <i>et al.</i> (2002); Foreman <i>et al.</i> (2004)
<b>Experimental studies using fumonisin-containing culture material</b>				
2 horses	Diet contained 19 ppm FB <sub>1</sub> and was fed ad libitum	27 days	None	Schumacher <i>et al.</i> (1995)
2 horses	Diet contained 200 ppm FB <sub>1</sub> and was fed ad libitum	12 and 16 days	Leukoencephalomalacia Increased GGT	Schumacher <i>et al.</i> (1995)
2 horses	2 diets containing 65 and 130 ppm FB <sub>1</sub> and were fed ad libitum	–65 ppm fed for 10 days –130 ppm fed for an additional 17 days	Leukoencephalomalacia Increased GGT	Schumacher <i>et al.</i> (1995)
<b>Experimental studies using naturally contaminated corn screenings</b>				
4 ponies	Diet contained 44 ppm FB <sub>1</sub> and was fed ad libitum	10 to 97 days	2 horses died with ELEM on days 10 and 45 – 2 horses were normal after 97 days	Wang <i>et al.</i> (1992)
4 ponies	Diets contained between 1 and 22 ppm FB <sub>1</sub> and were fed ad libitum	238 to 326 days	ELEM in 2 of 5 horses Moderate to mild hepatosis in all 5 horses	Wilson <i>et al.</i> (1992)
5 ponies	Diet contained 8 ppm FB <sub>1</sub> and was fed ad libitum	180 days	Mild histologic lesions in the brain and liver at necropsy	Wilson <i>et al.</i> (1992)
4 ponies	Diets contained between 1 and 88 ppm FB <sub>1</sub> and were fed ad libitum	9 to 120 days	ELEM in all 4 ponies (day 9 to 120) – also severe hepatosis in 2 ponies	Ross <i>et al.</i> (1993)
<b>Reported fumonisin concentrations from naturally occurring outbreaks</b>				
18 horses	37 to 122 ppm FB <sub>1</sub>	Unknown	ELEM confirmed in 14 horses	Wilson <i>et al.</i> (1990)
45 horses	8 to 126 ppm FB <sub>1</sub>	7 to 35 days	All cases had confirmed leukoencephalomalacia	Ross <i>et al.</i> (1991)
6 horses	370 ppm FB <sub>1</sub> and 105 ppm FB <sub>2</sub>	Unknown	4 horses died with ELEM; 2 horses with neurologic signs apparently recovered	Wilkins <i>et al.</i> (1994)
100+ donkeys	4 to 29 ppm FB <sub>1</sub>	Unknown	Many donkeys died of neurologic disease – ELEM confirmed in 3 cases	Rosiles <i>et al.</i> (1998)

hepatotoxicity (Osweiler *et al.*, 1993). Although it is tempting to speculate that cattle are able to break down the toxin, it has been shown that fumonisin is poorly metabolized by the rumen. In general, cattle have an increased tolerance to fumonisin because of differences in the mechanism of action. In milk-fed calves treated with purified fumonisin B<sub>1</sub>, the kidney was the target organ of toxicity (Mathur *et al.*, 2001). However, this

study also demonstrated that sphingosine and sphinganine concentrations did not increase in the serum and tissues of calves to the same degree that has been shown in pigs and horses.

When a group of 26 dairy cattle were fed a ration containing 100 ppm of fumonisin for the first 70 days of their lactation period, they had a significant decrease in dry matter intake and a lower milk yield as compared to the

control group (Diaz *et al.*, 2000). Milk production averaged 7kg lower in the group fed the ration containing fumonisin and there was a 13% decrease in feed intake over the duration of the study period. Therefore, it has been recommended to avoid fumonisin concentrations higher than 30 ppm in the total ration of dairy cattle.

Fumonisin toxicity in poultry

Fumonisin can be toxic to both chickens and turkeys with concentrations in the feed as low as 100mg/kg causing decreased body weight gain, diarrhea and hepatotoxicity (Ledoux *et al.*, 1992; Bermudez *et al.*, 1997). There has also been an association between *Fusarium verticillioides* (the fungus that produces fumonisin) and an acute death syndrome recognized in young chicks called spiking mortality syndrome. It was initially hypothesized that fumonisins were directly cardiotoxic to poultry and were the cause of this syndrome; however, more recent research has suggested moniliformin (another *F. verticillioides*-produced mycotoxin) is primarily responsible.

DIAGNOSIS AND TREATMENT

In addition to pathologic findings in animals, diagnosis of fumonisin toxicosis typically relies on detecting the actual toxin in feed samples. Fungal culture of feeds has little value in diagnosing fumonisin toxicosis because some corn samples contain very high concentrations of toxin with low levels of fungus, while other samples have heavy growths of *Fusarium* fungus with little to no detectable fumonisin. This is partly because the fungus that produced fumonisin also produces other mycotoxins. Therefore, the definitive diagnosis of fumonisin toxicosis in animals must involve analyzing the feed for the presence of the actual toxin. Many diagnostic laboratories across the world offer assays to detect both fumonisin B<sub>1</sub> and B<sub>2</sub> in corn and feed samples. The two most commonly used methods for toxin detection are chromatography (HPLC) and immunologic (ELISA) assays. To date, there are no commercially available assays that detect fumonisin in serum or tissues of animals.

Another assay that may be used more commonly in the future to diagnose fumonisin toxicosis in animals is the sphinganine to sphingosine ratio (Sa:So ratio). Because of the fumonisin-induced disruption of sphingolipid biosynthesis (Wang *et al.*, 1992), the Sa:So ratio increases in the serum and tissues of pigs and horses exposed to fumonisin. It has been suggested that this assay could be used to diagnose fumonisin toxicosis when feed analysis is not possible. Sphinganine and

TABLE 90.3 Recommended levels for total fumonisins (B<sub>1</sub> and B<sub>2</sub>) in animal feeds

Animal	Recommended maximum level of total fumonisins in corn to be used for feed (ppm)	Recommended maximum level of total fumonisin in the ration (ppm)
Horse <sup>a</sup>	5	1
Swine	20	10
Ruminants <sup>b</sup>	60	30
Poultry <sup>c</sup>	100	50
Ruminant and poultry breeding stock <sup>d</sup>	30	15
Catfish	20	10
Other animals <sup>e</sup>	10	5

From the United States Food and Drug Administration, Center for Veterinary Medicine.  
<sup>a</sup>Includes donkeys, asses and zebras.  
<sup>b</sup>Cattle, sheep, goats and other ruminants that are >3 months of age and are being fed for slaughter.  
<sup>c</sup>Turkeys, chickens, ducklings and other poultry being fed for slaughter.  
<sup>d</sup>Includes lactating dairy cows, bulls, laying hens and roosters.  
<sup>e</sup>Includes dogs and cats.

sphingosine data may be available from enough pigs and horses in the near future to provide values for normal and affected animals.

To date there have been no treatments described for either equine leukoencephalomalacia or porcine pulmonary edema. Generally the onset of clinical signs is acute and the progression of disease is rapid for both syndromes. The most important treatment is to identify and remove the source of contaminated feed to prevent other animals from developing clinical signs.

Guidelines for the maximum recommended levels of fumonisins in animal feeds have been published by the FDA Center for Veterinary Medicine (Table 90.3). It is important that livestock producers be aware of these guidelines and have their corn periodically tested for mycotoxins. Research has not yet found effective ways to decrease fumonisin concentrations in animal feed-stuffs through processing or feed additives (i.e., binding agents). Corn containing significant levels of fumonisin should be discarded, diluted with corn containing lower concentrations of fumonisin, or fed to a less-sensitive species (i.e., ruminants or poultry interested for slaughter).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Fumonisin remain an active research topic; however, most studies in the last few years have focused on the potential effects of this mycotoxin in humans. More



animal studies are needed to further define the mechanism of neurotoxicity in horses. Studies also need to be done examining the long-term cardiovascular effects associated with lower doses of fumonisin exposure in swine and humans. High fumonisin concentrations seem to appear in the corn crop from the United States every 3 to 4 years depending on weather, so they will continue to be a toxin of high regulatory concern in the future. Veterinarians and toxicologists must be familiar with this mycotoxin and should be able to quickly recognize clinical signs and gross lesions associated with fumonisin toxicity in animals.

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# Ochratoxins and citrinin

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## INTRODUCTION

Ochratoxins and citrinin are produced by several species of the genera *Aspergillus* and *Penicillium*. The two most common species that produce ochratoxin A (OTA) are *Aspergillus ochraceus* and *Penicillium verrucosum*. These fungi are ubiquitous and the potential for contamination of animal feed and human food is widespread. *Aspergillus* spp. appear to produce ochratoxins under conditions of high humidity and temperature, whereas some *Penicillium* spp. may produce ochratoxins at temperatures as low as 5°C. OTA has been found in a variety of food/feed, with levels in commodities used as feed ranging up to 27 ppm, and with levels in foodstuffs for human consumption in the range of trace to about 100 ppb. OTA is a pentaketide-derived dihydroisocoumarin moiety coupled with a 12-carboxy group by a peptide bond to *L*-phenylalanine. There are two commonly recognized OTA analogs, ochratoxin B (OTB) and ochratoxin C (OTC), and, of course, alkyl esters of ochratoxins. Unlike ochratoxin A (OTA), the occurrence of OTB and OTC is rare. Chemical structures of ochratoxins are shown in Figure 91.1 and the order of their toxicity is OTA > OTB > OTC. All metabolites of OTA are less toxic than the parent compound.

Citrinin was first isolated as a pure compound from a culture of *P. citrinum* in 1931. Later, it was also isolated from *A. ochraceus*, *P. verrucosum* and related species that contaminate grain. In 1951, yellowish colored rice imported from Thailand to Japan was found to be contaminated with *P. citrinum*, which contained citrinin. Synthesized citrinin is also used in molecular biological research, as it induces mitochondrial permeability

pore opening and inhibits respiration by interfering with complex I of the respiratory chain. The chemical structure of citrinin is shown in Figure 91.2.

Both OTA and citrinin cause nephropathy in animals and they have also been implicated as the cause of Balkan Endemic Nephropathy (BEN) in humans.

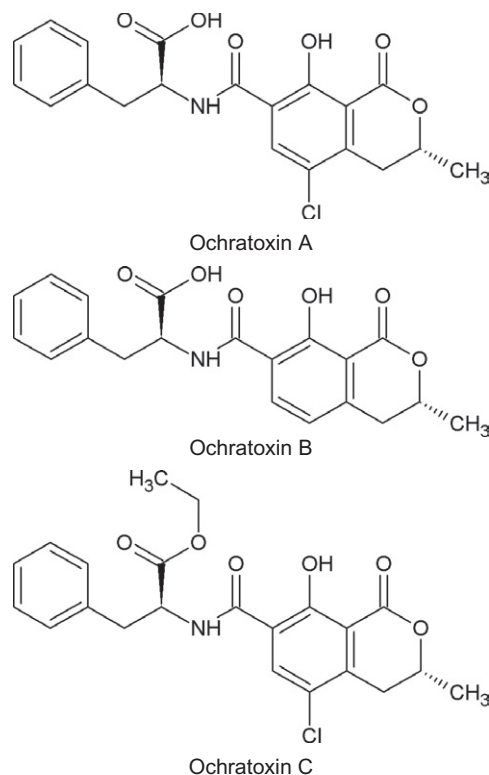


FIGURE 91.1 Chemical structures of ochratoxins.



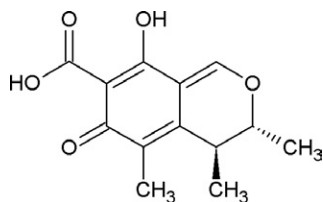


FIGURE 91.2 Chemical structure of citrinin.

Literature reveals that OTA has been studied to a greater extent than citrinin, because OTA is at least ten times more toxic than citrinin. This chapter describes in detail the toxicity of ochratoxins and citrinin in animals.

## BACKGROUND

The fungi producing ochratoxins and citrinin are commonly encountered in animal feed and human food around the world. They are encountered with great frequencies in the Balkan and Scandinavian countries. There are three major ochratoxins (OTA, OTB and OTC), but OTA occurs naturally with a greater frequency in a variety of cereal grains (barley, wheat, oats, corn and beans), peanuts, dried fruits, grapes/raisins, cheese and other food products. OTA accumulates in the food chain because of its long half-life. Citrinin usually co-occurs with OTA, and commonly contaminates cereal grains, including wheat, barley, oats, corn and rice. Citrinin also contaminates peanuts and fruits. Levels of OTA and citrinin have been found far lower in human food than in raw animal feed, because during the processing and baking of human food, citrinin is almost eliminated and OTA is significantly reduced. Compared to OTA, OTB and OTC are rarely found and are much less toxic.

Both OTA and citrinin are well-known nephrotoxins. OTA is also carcinogenic to rodents (Creppy *et al.*, 1985) and possesses teratogenic (Arora *et al.*, 1983), immunotoxic (Stormer and Lea, 1995), neurotoxic (Bruinink and Sidler, 1997; Sava *et al.*, 2006), mutagenic (Stetina and Votava, 1986) and genotoxic (Meisner *et al.*, 1983) properties. In humans, exposure to OTA and citrinin has been linked with Balkan Endemic Nephropathy (BEN), a chronic kidney disease associated with tumors of the renal system, which can be fatal. However, recent findings doubt OTA as the etiological factor in BEN (Mally *et al.*, 2007). Co-occurrence of citrinin with OTA has been implicated in nephropathy of pigs in Denmark, Sweden, Norway and Ireland. Citrinin and OTA are also involved in avian nephropathies. Residues of OTA have been detected in the tissues of pigs in slaughterhouses, and it has been shown, under experimental conditions,

that residues can still be detected in tissues 1 month after the end of exposure. Due to the long half-life of OTA in feed and biological systems, serious concerns have been raised about animal health as well as the human consumption of meat.

## TOXICOKINETICS

In most animal species, OTA is absorbed from the stomach because of its lipid soluble, non-ionized and acidic properties ( $pK_a = 7.1$ ). Absorption of OTA also takes place in the duodenum and jejunum, is involved in enterohepatic circulation and its biliary excretion is very efficient (Kumagai and Aibara, 1982; Kumagai, 1988). OTA is distributed to various organs, mainly to the kidneys. Liver, muscle and fat contain lower concentrations. Following oral administration, the overall percentage of OTA absorption is found to be 66% in pigs, 56% in rats, 56% in rabbits and 40% in chickens (Suzuki *et al.*, 1977; Galtier *et al.*, 1981). After a single oral dose, the maximum concentrations of OTA are found within 10–48 h in pigs and rats (Galtier *et al.*, 1979, 1981), 2–4 h in calves (Sreemannarayana *et al.*, 1988), after 1 h in rabbits and after 0.33 h in chickens (Galtier *et al.*, 1981). Maximum tissue concentrations in rat tissues occur within 48 h.

When absorbed, OTA has shown a high binding affinity for plasma proteins. OTA was found in decreasing order of concentrations in kidney < liver < fat < muscle. The serum half-life of OTA is long and varies widely among species, e.g., 24–39 h in mice, 55–120 h in rats, 6.7 h in quail, 510 h in *Macaca mulata* monkeys (Hagelberg *et al.*, 1989), 72–120 h in pigs, 4.1 h in chicken (Galtier *et al.*, 1981) and 840 h in a human (Benford *et al.*, 2001).

Toxicokinetics of OTA in pigs revealed that the kidney is generally the most heavily contaminated tissue and that levels in the blood are about five-fold greater than in the kidney. Krog *et al.* (1976) illustrated that if the level of OTA in swine kidney is 12.1 ng/g (resulting from about 1000 ng/g in the feed), its levels would be 7.8 ng/g in the liver, 4.2 ng/g in the muscle and 2.8 ng/g in adipose tissue. OTA in ruminants is usually hydrolyzed in the forestomach by protozoans and bacterial enzymes, and consequently little OTA is found in the tissues (Hult *et al.*, 1976).

In various tissues of all species that are examined, OTA is hydrolyzed to ochratoxin *alpha*, which is the major metabolite. This detoxication process takes place in the cecum of rats and is facilitated by bacterial microflora (Galteir, 1978). The enzymes responsible for hydrolysis to ochratoxin *alpha* in cows, sheep and rodents are carboxypeptidase A and chymotrypsin. Suzuki *et al.*

(1977) demonstrated that the rat tissue homogenate of the duodenum, ileum and pancreas also has a high activity of these enzymes to catalyze this reaction. Activity of these enzymes in liver and kidney are low. Studies in mice suggest that OTA circulates from the liver into the bile and into the intestine, where it is hydrolyzed to ochratoxin *alpha* (Moroi *et al.*, 1985). About 25–27% of OTA, given either i.p. or orally to rats, was found as ochratoxin *alpha* in the urine. Its presence in the urine can be explained by reabsorption from the intestine. A similar mechanism of intestinal reabsorption of ochratoxin *alpha* has been suggested to occur in ruminant calves (Sreemannarayana *et al.*, 1988). In sheep, Höhler *et al.* (1999) demonstrated that OTA hydrolysis in the gastrointestinal tract was substantially less than previously described, especially if OTA was ingested in combination with concentrate-rich diets.

OTA excretes in the urine and feces in all species (Höhler *et al.*, 1999; Ringot *et al.*, 2006; Carone *et al.*, 2011). OTA also passes in the milk in some species (such as rats and rabbits), but very little is excreted in cows' milk because of its metabolism by the ruminal microflora (Breitholtz-Emanuelsson *et al.*, 1993). This route is of importance because the milk is consumed by the offspring. Subsequent to its urinary excretion, OTA is reabsorbed in all nephron segments. Excretion of OTA can be influenced by the route of administration, the dose and the gender, age and weight of the animal (Vettorazzi *et al.*, 2009). Excretion of OTA is also impacted by the extent of enterohepatic circulation and binding to serum albumin and other macromolecules (Galtier *et al.*, 1980; Hult and Fuchs, 1986). The association constant for the binding of OTA to serum albumin is  $7.1 \times 10^4$  per mol for pigs,  $5.1 \times 10^4$  per mol for chickens and  $4.0 \times 10^4$  per mol for rats (Galtier *et al.*, 1981).

Placental transfer of OTA in mammalian species like mice, rat and swine is well known. However, in ruminants the placental transfer of OTA is very little. After intravenous administration of a high dose of OTA (1 mg/kg body weight) to pregnant ewes, Munro *et al.* (1973) did not detect OTA in the amniotic fluid and fetal tissue levels were 400 to 1000 times lower than in the maternal blood. The exact mechanism involved in placental transfer of OTA is yet to be elucidated. OTA can be present in eggs if the hens are exposed to this toxin at higher doses.

From animal studies, it is clear that OTA has a high degree of bioavailability, a low plasma clearance rate and a long tissue half-life. Because of the differences in animal physiology, wide variations are seen in the toxicokinetic patterns of OTA. For further details on biotransformation, toxicokinetics and toxicodynamics of OTA, readers are referred to a recent publication elsewhere (Ringot *et al.*, 2009). No toxicokinetic data of citrinin are available to describe in this chapter.

## MECHANISM OF ACTION

Both OTA and citrinin are well-known nephrotoxics. In addition to nephrotoxicity, OTA is known to exert neurotoxic, immunotoxic, teratogenic and carcinogenic effects in mammalian species. OTA also disrupts blood coagulation and glucose metabolism. A brief description of the mechanisms involved in common toxic effects is given below.

### Nephrotoxicity

Both OTA and citrinin produce nephrotoxicity involving multiple mechanisms. At high doses, OTA affects both renal function and morphology, as indicated by increased weight, urine volume, blood urea nitrogen, urinary glucose and proteinuria. The last two findings indicate that the site of reabsorption (i.e., the proximal convoluted tubules) is damaged. OTA specifically causes defect of the organic anion transport mechanism located on the brush border of the proximal convoluted tubules and basolateral membranes. OTA also adversely affects the organic ion transport system by which OTA enters the proximal tubular cells. The middle (S2) and terminal (S3) segments of the proximal tubule of the isolated nephron are the most sensitive to the toxic effects of OTA, as evidenced by marked decreases in cellular and mitochondrial ATP contents.

Studies suggest that both OTA and citrinin cause mitochondrial dysfunction in renal and hepatic tissues (Aleo *et al.*, 1991; Chagas *et al.*, 1995). OTA toxicity is associated with inhibition of both protein and RNA synthesis (Dirheimer and Creppy, 1991). OTA is known to interfere with the charging of tRNA with amino acids. OTA treatment can increase oxidative stress in peripheral organs. Administration of OTA to rats (1 mg/kg) resulted in a 22% decrease in  $\alpha$ -tocopherol plasma levels and a five-fold increase in the expression of the oxidative stress responsive protein heme oxygenase-1, specifically in the kidney (Gautier *et al.*, 2001). Cell death occurs by apoptosis.

### Neurotoxicity

Toxic effects of OTA on the CNS have not yet been fully characterized. Evidence strongly suggests that OTA affects selected structures of the brain and it has the potential for neurotoxicity (Belamadani *et al.*, 1998; Bruinink *et al.*, 1998). This mycotoxin has multiple mechanisms of action, including oxidative stress, bioenergetic compromise, inhibition of protein synthesis, production of DNA single-stranded breaks and formation of OTA-DNA adducts (Sava *et al.*, 2006). These authors found that administration of OTA in mice, at a single dose (3.5 mg/kg) that is approximately 10% of the reported LD<sub>50</sub>, caused widespread oxidative injury in six discrete brain regions.

## Immunotoxicity

There is ample evidence from studies conducted in several animal species that under certain conditions of treatment, OTA can produce defects in the structure and/or function of elements comprising the immune system (Pohland *et al.*, 1992). The size of the mouse thymus was reduced to 33% of that of controls after four i.p. injections of OTA at 20mg/kg body weight on alternate days, a dose which caused minimal nephrotoxicity. Bone marrow depression was dose related, and changes included decreased marrow cellularity, a reduction in bone marrow macrophage-granulocyte progenitors, a decreased number of hematopoietic stem cells, a significant decrease in erythropoiesis and increased phagocytosis by macrophages (Boorman *et al.*, 1984). The effects of OTA on the bone marrow and lymphatic cell population may reflect the sensitivity of these cells to the inhibition of protein synthesis. These effects on the structural components of the immune system indicated that OTA adversely affects immune function.

In chickens fed diets containing OTA at a concentration of 2–4mg/kg for 20 days, the lymphoid cell population of immune organs was decreased, and IgA and IgM in lymphoid tissues and serum were decreased (Dwivedi and Burns, 1984a,b). Complement activity was slightly affected in birds fed diets containing 2mg/kg for 5–6 weeks (Campbell *et al.*, 1983). Immune suppression was observed in chickens fed diets containing OTA at 0.05mg/kg or 2mg/kg for 21 days. Treated animals showed reduced total serum protein, lymphocyte counts and weights of the thymus, bursa of fabricus and spleen (Singh *et al.*, 1990).

## Carcinogenicity

The exact mechanism by which OTA induces nephrocarcinogenicity is yet to be established, although both genotoxic and non-genotoxic modes of action seem to be involved (Pfohl-Leschowicz and Manderville, 2007; Marin-Kuan *et al.*, 2008; Golli-Bennour *et al.*, 2010). Recently, Cavin *et al.* (2007) suggested that the reduction of antioxidant defense appears to be involved in OTA toxicity and carcinogenicity.

## TOXICITY

The toxic effects of ochratoxin A (OTA) have been studied extensively in a number of domestic, companion and experimental animals. All evidence suggests that OTA continues to be a huge problem to animal health worldwide (O'Brien and Dietrich, 2005). Overall toxicity of OTA is greatly influenced by species, sex and route of administration. Based on acute toxicity data, dogs, pigs

and chickens are the most sensitive species and rats and mice the least sensitive. Oral LD<sub>50</sub> values (expressed as mg/kg body weight) of OTA are reported to be 46–58 in mouse, 20–30 in rat, 3.9 in neonate rat, 0.2 in dog, 1 in pig and 3.3 in chicken (Harwig *et al.*, 1983). LD<sub>50</sub> values via i.p. route are reported to be 22–40 in mouse and 20–30 in rat; and with i.v. route, 26–34 in mouse and 13mg/kg in rat. OTA causes renal toxicity, nephropathy, neurotoxicity, immunotoxicity, carcinogenicity and developmental toxicity in several animal species.

The acute LD<sub>50</sub> (expressed as mg/kg body weight) of citrinin is reported to be 50 (oral) and 67 (s.c. or i.p.) in rat, 35–58 in mouse and 19 (i.p. or i.v.) in rabbit. Citrinin toxicity, especially in terms of nephropathy, has been reported in various animal species, including rats, hamsters, dogs and poultry. Citrinin causes kidney damage and mild liver damage in the form of fatty infiltration. Other toxic effects include vasodilatation, constriction of the bronchi and increased muscular tone.

All animals studied so far have been found susceptible to orally administered OTA with a varying degree of response. It is important to mention that at higher doses, OTA causes alterations in kidneys and also in other organs and tissue, but renal lesions can be found at an exposure level that is identical to those occurring environmentally. Ochratoxin B (OTB) is rarely found as a natural contaminant and is much less toxic than OTA. The other ochratoxins have never been encountered in natural products.

Weanling Fischer 344/N rats of both sexes receiving OTA by gavage in maize oil at a dose of 0.06, 0.12, 0.25, 0.5 or 1 mg/kg body weight/day for 5 days/week for 91 days, showed growth retardation and a reduced relative kidney weight in males at the two higher doses. Karyomegaly of dose-related severity was observed in the proximal tubules at all doses. Milder renal changes consisting of tubular atrophy were seen at a dose of 1, 4 or 16mg/kg body weight/day on 5 days/week for a total of 12 doses over 16 days. Rats receiving the highest dose had diarrhea and nasal discharge and died before the end of the study. Increased relative weights of kidneys, heart and brain, thymus atrophy, forestomach necrosis and/or hyperplasia, and hemorrhage of adrenal glands were seen at the two higher doses. Bone marrow hyperplasia and nephropathy were seen at all doses, involving renal tubular degenerative and regenerative changes (NTP, 1989).

OTA has been shown to produce nephrotoxic effects in all animal species examined, with the exception of adult ruminants (Pohland *et al.*, 1992). The nephrotoxic potential of OTA is well documented from all experimental studies, with a feed level of 200ppb causing nephropathy in pigs and rats. Evidence strongly supports that OTA is involved in porcine nephropathy, which is characterized by degeneration of the proximal tubules, atrophy of the tubular epithelium, interstitial fibrosis in the



renal cortex and hyalinized glomeruli. Field cases of OTA-induced nephropathy in farm animals have long been recognized. Benford *et al.* (2001) suggested that the adverse effect at the lowest effective dose in several mammalian species is nephrotoxicity. Citrinin is also nephrotoxic, but it is ten times less toxic than OTA.

In a series of experiments, sows were given feed containing OTA at a concentration of 0.2, 1 or 5 mg/kg (equivalent to 0.008, 0.04 and 0.2 mg/kg body weight/day), for a period of 5 days, 8 or 12 weeks, or up to 2 years. Decreased renal function, nephropathy and reduced renal enzyme activity were observed. Progressive nephropathy but no renal failure was seen in pigs given feed containing 1 mg/kg for 2 years (Krog *et al.*, 1976; Elling *et al.*, 1985).

Beagle dogs receiving OTA in capsule form at a dose of 0.1 or 0.2 mg/kg body weight/day for 14 days showed tubular necrosis and ultrastructural changes in the proximal tubules at all doses. Necrosis of lymphoid tissues of the thymus and tonsils was also seen at all doses (Kitchen *et al.*, 1977a,b,c). In another set of experiments, young beagle dogs were given OTA and citrinin separately and combined for 14 days (Kitchen *et al.*, 1977b). OTA was administered by capsule at 0.1 and 0.2 mg/kg; and citrinin (5 and 10 mg/kg) dissolved in ethanol was given by i.p. injection. Clinical signs of toxicosis with 10 mg/kg citrinin and the higher combined doses included anorexia, retching, tenesmus, weight loss, prostration and death. Severity of the clinical disease and mortality were increased when the mycotoxins were combined, which indicated synergism. The clinicopathological abnormalities reflected renal damage, cellular and granular casts and ketones as well as protein and glucose in the urine of dogs given large doses of citrinin alone or combination with OTA. In pathological studies, these authors found gross lesions, such as focal peritonitis and intestinal intussusceptions with citrinin. Changes in the kidneys of dogs given OTA were degeneration and necrosis with desquamation of tubular epithelial cells, primarily in the straight segment of the proximal tubules. Dogs given 10 mg/kg citrinin had similar changes in the distal tubules and collecting ducts. Dogs given combined doses of citrinin and OTA had degeneration and necrosis in proximal and distal tubules, and in thin segments and the collecting ducts and ulceration of the mucosa of the intestine.

In experimental studies, dogs given citrinin showed serous nasal discharge and lacrimation (Carlton *et al.*, 1974; Kitchen *et al.*, 1977a,b). It is important to mention that citrinin is a very strong emetic in dogs, which is a protective mechanism in this species. Therefore, it is very unlikely that dogs will be poisoned by citrinin alone because high amounts of this mycotoxin will induce emesis and feed refusal.

Chickens, turkeys and ducklings are all susceptible to OTA and it appears that OTA-contaminated feed

has a major economic impact on the poultry industry. Field cases of OTA-induced nephropathy are regularly encountered in poultry. Clinical signs of ochratoxicosis include retarded growth rate, reduction in weight gain, poor feed conversion, reduced egg production, poor egg shell quality, nephrotoxicity/nephropathy and mortality. Feed refusal has been observed in turkeys.

In chickens, OTA at a dose rate of 3.6 mg/kg can cause 5% mortality. Ochratoxin B at a dose rate of 54 mg/kg causes lowered growth rate, edema of visceral organs, and accumulation of uric acid in kidneys, liver, heart and spleen. These mycotoxins induce suppression of blood formation in bone marrow, and lymph formation in spleen and bursa of fabricus. The highest toxicity of OTA is found to be in broiler chickens. OTA given to broiler chickens at a dietary concentration of 4 mg/kg for 2 months caused a 42% mortality (Gibson *et al.*, 1990). This toxin is involved in reduced growth rate at 5 ppm, high mortality rate at 4–8 ppm and cessation of egg production at 4 ppm.

In chickens, nephrotoxicity and hepatotoxicity occur at dietary levels of 250 µg/g of citrinin with liver and kidney enlargements of 11 and 22%, respectively. Necropsy of affected birds revealed the presence of pale and swollen kidneys (Wyatt, 1979). Citrinin is at least ten times less nephrotoxic than OTA.

Griffiths and Done (1991) described an outbreak of citrinin toxicosis in a herd of cows which ingested citrus pulp (visibly moldy) pellets that contained 30–40 ppb citrinin. Affected cows showed signs of pruritis, pyrexia and hemorrhagic syndrome. Signs of the syndrome occurred within 3 days of ingesting the citrus pulp, which was fed for 21 days. Five calves whose dams had been fed citrus pulp were subsequently born with superior prognathism. Older animals were more susceptible to citrinin. The clinical signs, gross pathology and histology were suggestive of citrinin involvement. OTA has been well tested for carcinogenicity by oral administration in mice and rats. When OTA was administered in the diet, hepatocellular tumors (designated as well-differentiated trabecular adenomas), renal cell tumors (renal cystadenomas and solid renal-cell tumors), hepatomas (some exhibiting the trabecular structure) and hyperplastic hepatic nodules were observed in male mice (IARC, 1993). In another study, administration of OTA in the diet induced hepatocellular carcinomas and adenomas in female mice. Gavage administration of OTA to male and female rats resulted in a dose-related increase in the incidence of renal cell adenomas and adenocarcinomas. Furthermore, metastasis of the renal-cell tumors was also observed in male and female rats. OTA also increased the incidence and multiplicity of fibroadenomas of the mammary gland in female rats (NTP, 1989; IARC, 1993). In essence, these data suggest that OTA increases the incidence of hepatocellular tumors in mice of each sex and produces renal-cell adenomas in male



mice and in rats of each sex. Based on sufficient evidence of carcinogenicity in experimental animals, OTA is classified as a possible carcinogen in humans (Group 2B) (IARC, 1993). Citrinin has been demonstrated to be mutagenic in hepatocytes (Bailey *et al.*, 2002). There is limited evidence for the carcinogenicity of citrinin to animals.

It is well established that OTA and citrinin are reproductive and developmental toxicants. OTA is known to induce teratogenicity in mice, rats, hamsters, chicken and pigs. In the rodent fetus, OTA targets the CNS, but skeletal deformities are also observed. While the mechanism involved in OTA-induced teratogenesis remains unclear, it seems to directly affect both the progenitor cells and the embryo. Details of reproductive and developmental effects can be found in recent publications elsewhere (Gupta, 2009, 2011).

## TREATMENT

There is no specific antidote for ochratoxin(s) or citrinin toxicity. Recovery is usually slow. Immediate removal of the suspected feed and replacement with clean feed supplemented with increased vitamin levels can be rewarding. Growth of *A. ochraceus* in a common food, such as cereals, can be controlled or minimized by drying them rapidly and thoroughly. Effective approaches to grain storage include fumigation, aeration and cooling, sealed storage, and controlled atmosphere in tropical and subtropical regions where insect damage is a major problem. Citrinin is less of a problem because it is heat unstable. Citrinin is also likely to be destroyed during brewing. Propionic acid, added as a preservative to protect barley in the feed from fungi, destroys citrinin during storage. Currently, highly sophisticated methods are available to detect these mycotoxins at ppb or lower levels in food/feed or their products, so as to prevent animal health from toxicosis and economic loss.

## CONCLUSIONS

Ochratoxin A (OTA) and citrinin both contaminate a wide range of animal feed and human food. Human risk is lower because the levels of these mycotoxins are minimized during processing and baking, but the raw feed remains a potential source for animal poisoning. Because of its long half-life, OTA accumulates in the body. These mycotoxins have a serious impact on the health of animals, especially pigs, dogs and poultry. Pigs and dogs are most sensitive, while rats and mice are least sensitive. In general, females are more sensitive than males.

The kidney is a major target organ (as evidenced by functional and morphological changes) for both mycotoxins, but other organs are affected as well. OTA and its analogs can produce a variety of toxic effects, referred to as "ochratoxicosis," including mutagenesis, carcinogenesis, embryotoxicity, teratogenesis and immune suppression, by damaging mitochondria, DNA, protein and RNA by lipid peroxidation and oxidative injury. Cell death occurs by apoptosis. Citrinin produces toxic effects similar to OTA but the severity is at least ten times less.

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# Slaframine

Geof W. Smith

## INTRODUCTION

Slaframine is an alkaloidal mycotoxin produced by the fungus *Rhizoctonia leguminicola* which causes profuse salivation (“slobbers”) in animals. *R. leguminicola* is a common fungal pathogen of red clover (*Trifolium pratense*) and causes a syndrome known as black patch disease in the plant. Ingestion of clover hay containing slaframine causes salivary episodes that last from several hours to over 3 days in ruminants and horses. Although the disease is short term and animals generally recover without treatment, the dramatic clinical signs associated with slaframine ingestion make it readily apparent to livestock owners. Diagnosis can be made by identification of *R. leguminicola* in suspect forage or by the detection of slaframine in plasma samples from exposed animals.

## BACKGROUND

Outbreaks of profuse salivation in cattle were initially reported in the late 1940s and 1950s from agricultural experiment stations in the Midwestern United States (O'Dell *et al.*, 1959). Most of these cases were associated with the feeding of second-cutting red clover hay. In 1956 it was first reported that fungal contamination of red clover with *R. leguminicola* was associated with a pasture disease called black patch, which derives its name from the appearance of affected areas in the field and not the characteristic black lesions on the leaves of affected plants (Croom *et al.*, 1995). Although its primary host is red clover, black patch disease has been

reported in other legumes including white clover, soybeans, kudzu, cowpea, blue lupine, alsike clover, alfalfa, lespedeza and milk vetch (Smalley and Sanderson, 1993). However, in most of these cases, infected red clover plants were present in the same areas of the field. Fungal infestations are usually associated with periods of wet weather and high humidity (Croom *et al.*, 1995). Transmission is thought to be primarily seed borne as the fungus overwinters on contaminated hay and can survive at least 2 years on contaminated seed.

## CHEMISTRY AND TOXICOKINETICS

Several laboratories were able to isolate the “slobber-causing” agent in *R. leguminicola*-contaminated red clover (Aust and Broquist, 1965; Rainey *et al.*, 1965) and in 1968 the chemical structure was described as 1-acetoxy-6-amino-8-hydroxyindolizidine (Gardiner *et al.*, 1968). The term slaframine became the most commonly used word for describing this compound (Aust *et al.*, 1966). Slaframine is a piperidine or indolizidine alkaloid (Figure 92.1) with the empirical formula  $C_{10}H_{10}N_2O_2$ , which gives it a molecular weight of 198 daltons (Croom *et al.*, 1995).

Slaframine is activated by hepatic microsomal enzymes into a ketoimine that is required to produce clinical signs (Figure 92.1). In cattle, the onset of salivation is shorter with more direct routes of administration; with intravenous injections producing salivation faster than intraperitoneal or intramuscular routes which are in turn faster than the subcutaneous route (Croom *et al.*, 1995). It has been suggested that slaframine is

metabolized in the liver by a microsomal flavoprotein oxidase to the ketoimine metabolite (Guengerich and Aust, 1977) consisting of a quaternary nitrogen separated from an acetate ester by two carbon atoms (Figure 92.1). This structure is very similar to that of acetylcholine, a parasympathetic neurotransmitter.

## MECHANISM OF ACTION

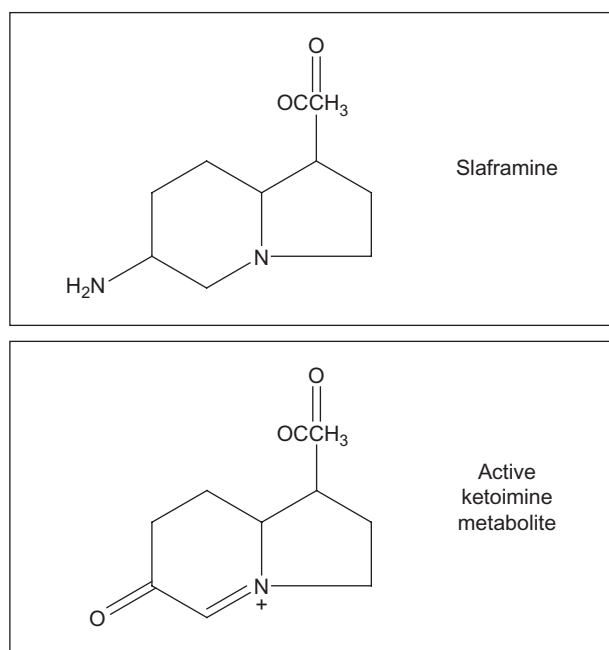
Pharmacologically, slaframine would be classified as a cholinergic agonist and/or a parasympathomimetic chemical. The majority of the available data indicate that the clinical signs produced by slaframine are due to its high affinity for the  $M_3$  muscarinic receptor subtype which is believed to be important in the control of exocrine and endocrine glands (Croom *et al.*, 1995). Early studies demonstrated that the increased salivation associated with slaframine toxicity could be blocked with pre-administration of atropine (Aust, 1970). Additionally, mortality in broiler chicks when slaframine is dosed at  $LD_{50}$  can be significantly decreased by the pre-administration of the muscarinic receptor antagonists atropine (which has affinity for  $M_1$ ,  $M_2$  and  $M_3$  receptors) and pirenzepine ( $M_1$  and  $M_2$  selective), but not gallamine which is  $M_2$  selective (Croom *et al.*, 1995). The pre-administration of the  $M_3$  selective antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4DAMP)

can also block the increase in pancreatic fluid output induced by slaframine (Walker *et al.*, 1994). Furthermore, slaframine has no effect on the cardiovascular system or arterial pressure at doses stimulatory to exocrine glands (Aust *et al.*, 1968), nor does it inhibit blood cholinesterase activity (Crump *et al.*, 1967; Hagler and Croom, 1987).

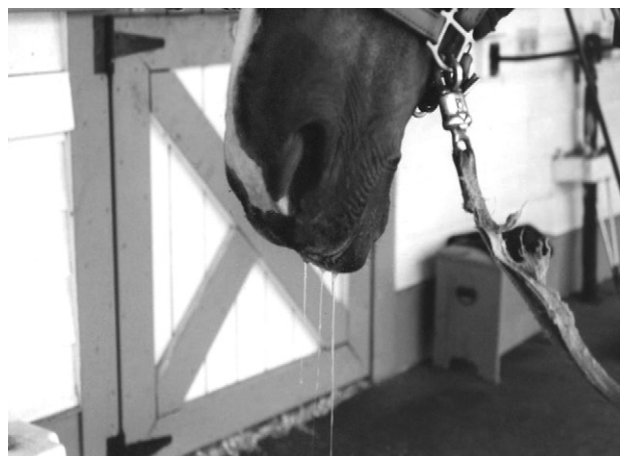
The stimulation of  $M_3$  muscarinic receptors by slaframine produces a profound stimulation of exocrine glands, particularly the salivary gland and pancreas. Steers given an intramuscular administration of purified slaframine had resting salivary flow rates 50 to 70% greater than saline-treated controls (Froetschel *et al.*, 1986). Similar findings documenting increases in saliva production both in cattle and sheep have been reported in other studies as well (Bird *et al.*, 1993; Hibbard *et al.*, 1995). Additional reported physiological effects of slaframine include increased pancreatic enzyme secretion (Aust *et al.*, 1968, 1970), increased rumen pH and rate of nutrient passage, and decreased ruminal motility in cattle (Croom *et al.*, 1995).

## TOXICITY

Clinical signs of slaframine toxicity are similar in all species. Following exposure to contaminated forages, animals begin to salivate profusely. Experimentally, a single dose of slaframine produces salivation for 6 to 10 hours. However, clinically affected animals can "slobber" for several days, presumably because they have continued access to slaframine contaminated forages (Figure 92.2). Other clinical signs can include anorexia, diarrhea, frequent urination and bloat. Decreased milk production can be expected in dairy cattle, likely related



**FIGURE 92.1** The structures of slaframine and the active ketoimine metabolite.



**FIGURE 92.2** A horse showing profuse salivation from ingesting slaframine-contaminated red clover hay (photo courtesy of Dr. Cecil Brownie, North Carolina State University).



to a decrease in feed intake (Crump, 1973). Although slaframine toxicosis has been experimentally induced in a variety of species including swine, poultry, cats, dogs, guinea pigs and rodents, naturally occurring cases are primarily reported in horses and ruminants (Crump *et al.*, 1967, 1995; Sockett *et al.*, 1982; Wijnberg *et al.*, 2009).

Cyanosis and open-mouth breathing have been reported under experimental conditions in sheep, swine and guinea pigs. Pigs also were observed to vomit, became dyspneic and collapsed with stiffened pelvic limbs (Crump *et al.*, 1967). Only mild salivation was noted in a small chicken fed slaframine and clinical signs resolved within a few hours. The LD<sub>50</sub> in day-old broiler chicks was estimated at approximately 81.6 mg/kg of body weight (Croom *et al.*, 1995), but higher doses (250–300 mg/kg) were required to produce death in guinea pigs (Crump *et al.*, 1967). Gross lesions in these animals consisted of vascular congestion of the thoracic and abdominal cavities. Pulmonary edema, disruption of the alveolar structure, emphysema and hepatic centrilobular necrosis were noted histologically. Death in these animals was attributed to suffocation from pulmonary edema and/or emphysema.

The clinical signs associated with the “slobbers syndrome” as described under field conditions include salivation, lacrimation, feed refusal, bloating, stiff joints, diarrhea and weight loss. However, very few of these signs have been seen following the administration of purified slaframine under experimental conditions. That has led to speculation that the alkaloid swainsonine might also be involved in producing clinical signs in classic slaframine toxicity (Croom *et al.*, 1995). Swainsonine is another alkaloid produced by *R. leguminicola* and has a similar structure to slaframine. Although it is better known for its association with locoweed toxicity, it is possible swainsonine is partially

responsible for some of the clinical signs classically observed with the “slobbers syndrome.”

## DIAGNOSIS AND TREATMENT

Diagnosis of slaframine toxicity is generally made by observation of clinical signs (salivation) in animals consuming legume forage, particularly red clover hay. Further tests can identify the fungus *R. leguminicola* in the hay which usually can be easily isolated in culture (Figure 92.3). Although chemical analysis for slaframine is not usually necessary, chromatographic methods for detecting the toxin in hay, plasma or milk have been described (Hagler and Croom, 1989; Imerman and Stahr, 1998).

Treatment is not usually indicated as animals typically recover spontaneously when the contaminated hay is removed; however, clinical signs may persist for 1 to 2 days following removal of the toxic forage. In severe cases, atropine may be of benefit to reverse the parasympathomimetic effects of slaframine; however, it is unlikely to completely resolve clinical signs. In guinea pigs, simultaneous administration of atropine and a lethal dose of slaframine prevented clinical signs for 5 hours, after which mild salivation was observed. When atropine was given 2 to 4 hours after slaframine administration, profuse salivation was observed; however, there was no mortality (Crump *et al.*, 1967). Atropine should be used with caution in ruminants and horses because of possible gastrointestinal side effects. Therefore treatments other than removing the contaminated hay are not usually recommended in affected animals.

Control of black patch disease remains a major problem for agronomists. Fungicides applied before flowering do not reduce seed infection, and ground sprays at



**FIGURE 92.3** Two photomicrographs of *Rhizoctonia leguminicola* mycelia growing in culture – 200× magnification – note the normal difference in pigment color between the two isolates (photos courtesy of Dr. Paul Vincelli and Cheryl Kaiser, University of Kentucky).

the time of plant growth in the spring and/or immediately after the first hay cutting have not been shown to reduce fungal contamination. Prevention requires selecting *R. leguminicola* varieties that are less susceptible to fungal infection and chemically treating seed prior to planting. Feasible mechanisms to detoxify or degrade the toxin in pasture and/or hay have not been found. Therefore control of slaframine outbreaks rely on completely replacing contaminated forages.

## CONCLUSIONS

There has not been any active research on slaframine in the last 10–20 years; however, clinical cases still occur relatively frequently. It is important for veterinarians and toxicologists to be familiar with this mycotoxin, to recognize the likely cause of profuse salivation in horses and cattle consuming red clover hay and to be able to make appropriate recommendations for treatment and control.

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# Tremorgenic mycotoxins

Timothy J. Evans and Ramesh C. Gupta

## INTRODUCTION

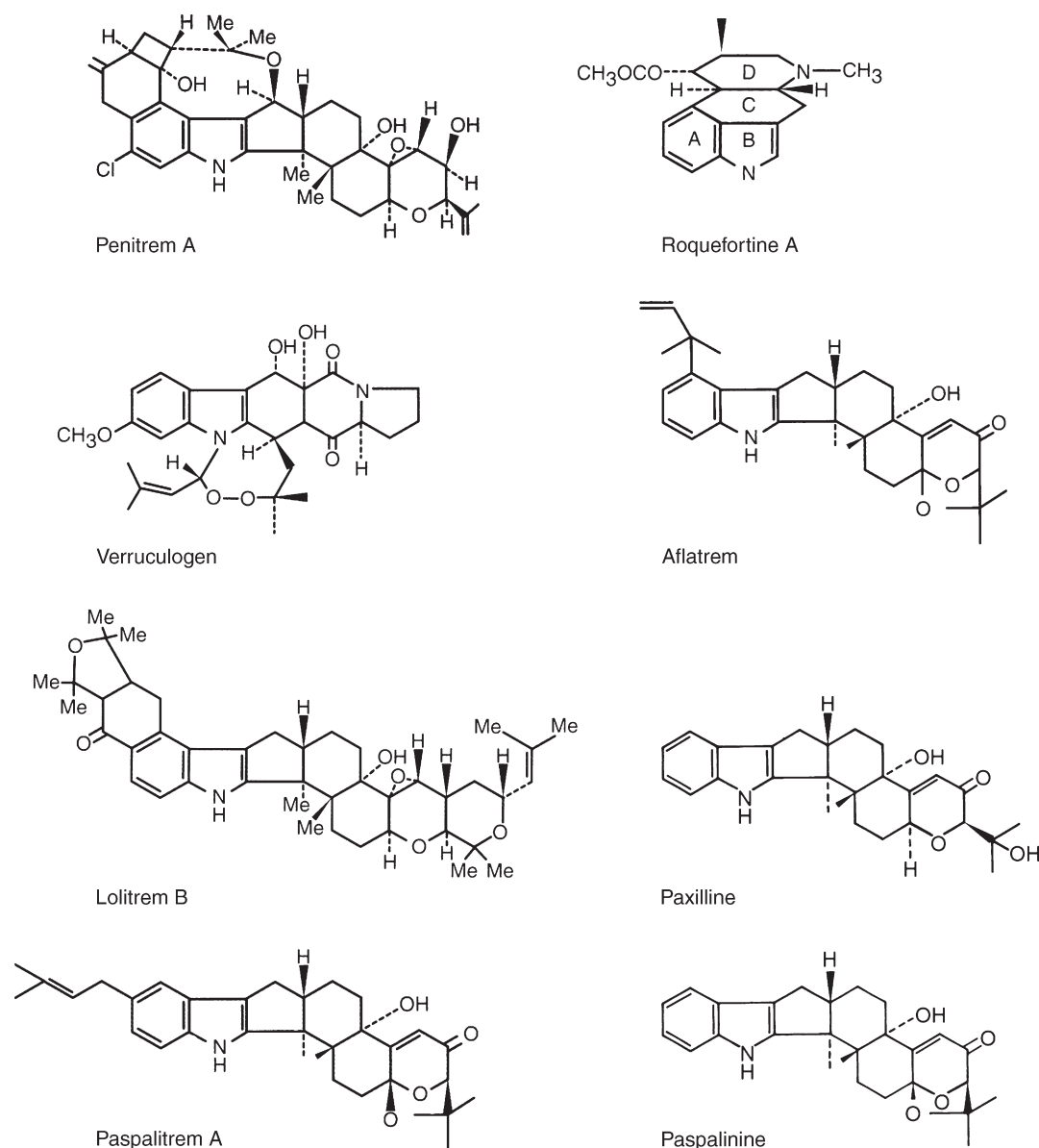
Fungi belonging to the genera *Penicillium*, *Aspergillus*, *Claviceps* and *Neotyphodium* can produce tremorgenic mycotoxins, which are secondary fungal metabolites that elicit either intermittent or sustained tremors in vertebrate species (Cole and Cox, 1981; Selala *et al.*, 1989; Burrows and Tyrl, 2001). Over 20 mycotoxins containing a tryptophan-derived indole moiety, including penitrems, roquefortine A, verruculogen, tryptoquivaline, aflatrem, paspalinine and paxilline, as well as the paspalitrems and lolitrems (Table 93.1 and Figure 93.1), have demonstrated tremorgenic potential in animals and humans (Selala *et al.*, 1989; Burrows and Tyrl, 2001). Several other fungal metabolites, such as roquefortine C, paspaline, paspalanine and cyclopiazonic, are chemically related to these mycotoxins but have been shown to not be tremorgenic (Knaus *et al.*, 1994; Tiwary *et al.*, 2009). With increased analytical capabilities, particularly the emergence of advanced NMR, HPLC-MS and LC-MS/MS spectroscopic methodologies, other “known” mycotoxins, with both related and unrelated structures, such as ergonovine (ergometrine), ergine (lysergic acid amide) and patulin, as well as “new” secondary fungal metabolites, like seco-penitrem D, have recently been detected in samples associated with clinical cases of tremogenic syndromes and their associated neurotoxicities (Sabater-Vilar *et al.*, 2004; Uhlig *et al.*, 2009; Moldes-Anaya *et al.*, 2011). However, the precise roles of these detected fungal metabolites in the pathogenesis of the observed tremogenic syndromes are yet to be determined, and this task is complicated by the diversity of fungal matrices, the propensity for myco- and

TABLE 93.1 Representative tremorgenic mycotoxins, associated fungi and substrates

Tremorgenic mycotoxin	Associated fungi	Common substrates
Penitrem A	<i>Penicillium crustosum</i> <i>Penicillium cyclopium</i> <i>Penicillium commune</i> <i>Penicillium</i> spp.	Meat, cereals, nuts, cheeses, eggs, fruits, processed/refrigerated foods, refuse, compost
Roquefortines <sup>1</sup>	<i>Penicillium roqueforti</i> Same as penitrem A and sometimes concurrently with penitrem A	Same as penitrem A
Janthitrems A, B and C	<i>Penicillium janthinellum</i>	Perennial ryegrass
Verruculogen	<i>Penicillium</i> spp. <i>Aspergillus</i> spp.	Soil, seeds, cereal crops
Tryptoquivaline tremorgens <sup>2</sup>	<i>Penicillium</i> spp. <i>Aspergillus clavatus</i>	Soil, seeds, cereal crops Sprouting cereal grains, malting byproducts
Territrems A and B	<i>Aspergillus terreus</i>	Cereal grains
Aflatrem	<i>Aspergillus flavus</i>	Corn
Lolitrems A, B, C and D	<i>Neotyphodium lolii</i>	Perennial ryegrass
Paxilline	<i>Neotyphodium lolii</i>	Perennial ryegrass
Lolitriol	<i>Neotyphodium lolii</i>	Perennial ryegrass
Paspalitrems A, B and C	<i>Claviceps paspali</i> <i>Claviceps cinerea</i>	Dallisgrass Bahia grass <i>Hilaria</i> spp.
Paspalinine	<i>Claviceps cynodontis</i> Same as paspalitrems	Bermudagrass Same as paspalitrems

<sup>1</sup>Roquefortine C has actually been associated with paralysis in several species.

<sup>2</sup>Includes quinazoline ring-containing indole alkaloids with structures similar to tryptoquivaline.



**FIGURE 93.1** The structures of selected indole-diterpene tremorgenic mycotoxins are shown. This figure was adapted, with permission, from CAST (2003) and from [Cole and Cox \(1981\)](#) (modifications courtesy of Don Connor and Howard Wilson).

phytotoxins to be present in complex mixtures, and difficulties inherent to relating xenobiotic structure to function and concentration to biological relevance.

Tremorgen-producing fungi grow on a wide variety of foodstuffs, including dairy or grain-containing products intended for human consumption (e.g., cheeses and pastas), stored grains and nuts (e.g., peanuts and walnuts) and a number of forages (e.g., legumes and grasses) consumed by livestock species, and even food or beverage manufacturing byproducts, garbage and compost piles can be sources of tremorgenic mycotoxins ([Burrows and Tyl, 2001](#); [Boysen et al., 2002](#); [Yount et al., 2003](#)). Although there have been several human cases

in which tremors and convulsions were attributed to consumption of mold-contaminated food containing penitrem A ([Gordon et al., 1993](#); [Lewis et al., 2005](#)), this chapter will focus on the toxicity of tremorgenic mycotoxins in animal species. Dogs, because of their relatively indiscriminate appetite and frequently unsupervised roaming behavior, appear to be very susceptible to intoxication by penitrem A, as well as roquefortines ([Boysen et al., 2002](#); [Yount et al., 2003](#)). Mycotoxin-associated stagger syndromes in livestock, also described in the literature as “grass staggers” (not to be confused with “grass staggers” or “grass tetany” related to hypomagnesemia), have most frequently occurred following the ingestion



of endophyte (*Neotyphodium lolii*)-infected perennial ryegrass (*Lolium perenne*) or consumption of Dallisgrass (*Paspalum dilatatum*) or Bahiagrass (*Paspalum notatum*) contaminated by sclerotia of *Claviceps paspali*.

## BACKGROUND

### *Penicillium*-associated tremorgenic mycotoxins

Although a variety of different fungi synthesize indole-diterpene mycotoxins having specific tremorgenic effects on the central nervous system (CNS), tremorgens produced by *Penicillium* spp. (especially penitrem A and roquefortines) are the most commonly encountered of these mycotoxins. Penitrem A is a potent neurotoxin which causes a syndrome characterized by sustained tremors and, at high doses, convulsions and death in laboratory and farm animals (Wilson, 1971; Arp and Richard, 1981; Peterson and Penny, 1982; Shreeve *et al.*, 1983; Hocking *et al.*, 1988; Breton *et al.*, 1998; Cavanagh *et al.*, 1998). Dogs can be poisoned by eating walnuts and a variety of other unused or discarded foodstuffs infected with *Penicillium* spp. (Boysen *et al.*, 2002; Yount *et al.*, 2003), and field cases of poisoning have also been documented in cattle, sheep and horses (Cavanagh *et al.*, 1998; Hocking *et al.*, 1988; Boysen *et al.*, 2002; Walter, 2002; Yount *et al.*, 2003). *Penicillium crustosum* is an especially common foodborne fungus that causes spoilage in a wide variety of foods, including meat, cereals, nuts, cheese, eggs, fruits and processed and refrigerated food, and almost all *Penicillium crustosum* isolates produce the mycotoxin penitrem A (Hocking and Pitt, 2003; Rundberget *et al.*, 2004). Roquefortines can also be synthesized by *Penicillium* spp. (Hooser and Talcott, 2006), and, interestingly, *Penicillium crustosum*, as well as several other *Penicillium* spp., can produce penitrem A and roquefortines concurrently following growth and sporulation (Vesonder *et al.*, 1980; Wagener *et al.*, 1980; Kyriakidis *et al.*, 1981; Mantle *et al.*, 1983; Boysen *et al.*, 2002; Yount *et al.*, 2003). In fact, roquefortine C, which has itself been associated with anorexia and paralysis, has been found to be a reliable biomarker for the tremorgenic mycotoxin penitrem A, in instances where that tremorgen was present in lower concentrations, at or slightly below its level of detection (Tiwary *et al.*, 2009). Additionally, three other tremorgenic mycotoxins, janthitrem A, B and C (molecular weight 601, 585 and 565, respectively), can also be produced by a species of *Penicillium* (i.e., *Penicillium janthinellum*), and these tremorgens have been associated with staggers outbreaks in sheep grazing ryegrass (Gallagher *et al.*, 1980; Burrows and Tyrl, 2001).

### *Aspergillus*-associated tremorgenic mycotoxins

Verruculogen, another tremorgenic mycotoxin containing the indole moiety and associated with tremors in mice, rats and farm animals, is produced by species of *Aspergillus*, as well as *Penicillium* (Gallagher and Latch, 1977). Likewise, tryptoquivaline and related quinazoline ring-containing indole alkaloids are also produced by *Aspergillus* and *Penicillium* spp. (Gao *et al.*, 2011). Tremorgenic mycotoxins isolated from *Aspergillus terreus* include territrems A and B, which were previously designated as C<sub>1</sub> and C<sub>2</sub>, respectively (Ling *et al.*, 1979), and *Aspergillus flavus* can, in addition to aflatoxins, also produce tremorgenic aflatrems and other indole-diterpene mycotoxins (e.g., paspalinine) in contaminated corn (Burrows and Tyrl, 2001).

### *Neotyphodium*-associated tremorgenic mycotoxins

In Australia, New Zealand and North America, perennial ryegrass (*Lolium perenne*) infected with the endophyte *Neotyphodium lolii* (formerly *Acremonium lolii*) has been associated with a “grass staggers” syndrome in horses, deer, cattle and, especially, sheep, which is frequently referred to as “perennial ryegrass staggers” and is distinct from annual ryegrass toxicosis (Galey *et al.*, 1991; Cheeke, 1998; Burrows and Tyrl, 2001). *Neotyphodium lolii* concentrates in the caryopsis/seed and the outer, lower leaf sheaths of perennial ryegrass and the “staggers” syndrome is most often observed after several days of exposure to endophyte-infected ryegrass during late summer when there is shortage of pasture (Galey *et al.*, 1991; Cheeke, 1998). Ergovaline and other ergot alkaloids associated with “fescue toxicosis” are also produced by *Neotyphodium lolii* (Cheeke, 1998), but the classic neurological signs of perennial ryegrass staggers are indistinguishable from those associated with *Penicillium* and *Aspergillus* species and the adverse effects of neurotoxic indole-diterpene tremorgens, including lolitrems A, C and D, lolitrem precursors (e.g., paxilline and lolitriol) and, especially, lolitrem B (Cheeke, 1998; Burrows and Tyrl, 2001).

### *Claviceps*-associated tremorgenic mycotoxins

While *Claviceps purpurea* is notoriously associated with “classic” ergotism in human and animals and, historically, occasional tremors or convulsions (i.e., nervous ergotism or convulsive ergotism) in livestock (Burrows and Tyrl, 2001; Evans *et al.*, 2004), other species of *Claviceps* produce toxicoses primarily characterized by a tremorgenic “staggers” syndrome (Burrows and Tyrl, 2001). Sheep, horses and cattle, in particular, can develop

a “grass staggers” syndrome several days following the ingestion of mature Dallisgrass (*Paspalum dilatatum*) or Bahiagrass (*Paspalum notatum*) infected with the sclerotia of *Claviceps paspali* (Cheeke, 1998; Burrows and Tyrl, 2001). “Dallisgrass staggers” and “Bahiagrass staggers” have most frequently been observed in the southeastern United States, Central and South America, parts of Europe and South Africa, as well as Australia and New Zealand. Traditionally, the ergot alkaloids produced in large quantities by *Claviceps purpurea* and in much smaller quantities by *Claviceps paspali* were thought to be responsible for the tremors observed in conjunction with exposure to both species of *Claviceps* (Cheeke, 1998). However, it is now understood, at least with respect to the “staggers” syndromes associated with *Claviceps paspali* and, less commonly, *Claviceps cinerea* (fungal infections of *Hilaria* spp. (curly mesquite, curlygrass, galleta, etc.)), that the large concentrations of indole–diterpene tremorgenic mycotoxins (i.e., paspalinine and paspalitrems A, B and C) found in the sclerotia of these species of *Claviceps* are responsible for the neurotoxicity. As with other “grass staggers,” *Claviceps*-related tremorgenic syndromes are characterized by exercise-exacerbated nervousness, “wild” facial expressions, belligerent attitude, tremors, ataxia, convulsions and occasional deaths attributed primarily to misadventure (Burrows and Tyrl, 2001).

### Bermudagrass staggers

Periodic episodes of tremors have been observed in cattle in the southern United States and Oklahoma and Texas, as well as horses in California, which have been ingesting mature Bermudagrass (*Cynodon dactylon*) (Cheeke, 1998; Burrows and Tyrl, 2001). Ergot-type alkaloids (ergine, ergonovine and ergonovinine) are produced by several species of *Claviceps*, including *Claviceps cynodontis*, and have been isolated in several instances of “Bermudagrass staggers” (Cheeke, 1998; Burrows and Tyrl, 2001; Uhlig *et al.*, 2009). However, higher concentrations of paspalitrem-type indole alkaloids have recently been detected in samples from a clinical case of this disease syndrome and are most likely the primary Bermudagrass tremorgens (Uhlig *et al.*, 2009).

## TOXICOKINETICS

It should be kept in mind that, while the onset and duration of the various “staggers” syndromes might vary somewhat, the tremorgenic neurotoxicities associated with the various grasses and aflatoxin-infected corn are essentially identical to one another (Cheeke, 1998). Tremorgenic indole-containing mycotoxins are lipophilic molecules that

easily cross the blood–brain barrier and rapidly gain access to the CNS (Patterson *et al.*, 1981). Although there might be some subtle differences between compounds and species of animals, tremorgenic indole–diterpene alkaloids are generally rapidly absorbed from the gastrointestinal tract, and the toxic signs, depending on the specific toxins and the exposed species, are usually seen within several hours (especially penitrem A and roquefortines in dogs) to a few days (frequently lolitrems and paspalitrems in ruminants) following ingestion of contaminated foodstuffs (Burrows and Tyrl, 2001; Hooser and Talcott, 2006). As evidenced by the lack of a cumulative effect following repeated doses, these particular mycotoxins do not appear to accumulate in the body (Peterson and Penny, 1982). Despite some hepatic metabolism, these mycotoxins are usually eliminated mainly through biliary excretion into the feces (Burrows and Tyrl, 2001; Hooser and Talcott, 2006). Young animals are more sensitive than adults to the toxicity of tremorgenic mycotoxins, with 5-month-old lambs being more susceptible than 15-month-old sheep to verruculogen toxicity (Patterson *et al.*, 1981).

## MECHANISM OF ACTION

It should be pointed out that, although related in structure, tremorgenic mycotoxins do not exert toxicity by a single common mechanism, and the exact mechanisms involved in neurotoxicity induced by these tremorgens are yet to be fully elucidated. These mycotoxins work at a different functional level of the nervous system than other mycotoxins having more widespread targets for toxicity associated with the inhibition of basic cellular functions, such as protein synthesis. Several pharmacological/toxicological mechanisms of tremorgenic action have been proposed, and, in general, tremorgenic mycotoxins interfere with inhibitory neuroreceptors and enhance excitatory amino acid neurotransmitter release mechanisms (Wilson, 1971; Norris *et al.*, 1980; Selala *et al.*, 1989). In experimental studies, the inhibitory action of this type of mycotoxin at gamma aminobutyric acid (GABA) receptors was demonstrated (Stern, 1971; Hotujac and Stern, 1974; Hotujac *et al.*, 1976; Selala *et al.*, 1989; Abramson, 1997). Gant *et al.* (1987) studied the effects of four fungal tremorgens and one nontremorgenic mycotoxin on GABA<sub>A</sub> receptor-binding in the rat’s brain, and, in high concentrations, the tremorgenic action of these mycotoxins was, in part, most likely due to inhibition of GABA<sub>A</sub> receptor function. Conversely, other studies have shown how tremorgenic mycotoxins increase the release of excitatory neurotransmitters (Norris *et al.*, 1980).

Among the tremorgenic mycotoxins, penitrem A has been studied the most for the elucidation of its mechanism

of action and toxicity. There is convincing evidence suggesting that penitrem A acts on the CNS to induce seizures (Sobotka *et al.*, 1978; Arp and Richard, 1981). Penitrem A was shown to increase the spontaneous release of aspartate and glutamate, as well as, interestingly, GABA (Norris *et al.*, 1980). Glutamate and aspartate are the neurotransmitters of the parallel and climbing fibers, respectively, which are two major excitatory inputs to Purkinje cells. There is also evidence for a penitrem A-induced partial decrease in glycine levels in the brain in association with pathology related to penitrem A exposure (Catovic *et al.*, 1975). Cavanagh *et al.* (1998) demonstrated that, in rats, penitrem A can cause widespread degeneration of Purkinje cells and foci of necrosis in the cerebellar granular cell layers. These striking lesions are confined to the cerebellum, with no lesions found elsewhere in the brain. Cerebellar Purkinje cells are the primary targets cells for the adverse effects of penitrem A. Penitrem A-induced tremors might also be partly explained by a presynaptic inhibition of inhibitory interneurons.

Similar mechanisms of action have been demonstrated for other tremorgenic mycotoxins. Verruculogen-induced tremors in rats have been associated with an increased level of excitatory neurotransmitters (glutamate and aspartate) in the lateral ventricle, suggesting subcortical, verruculogen-induced tremorgenic activity. Selala *et al.* (1989) demonstrated that tremorgenic mycotoxins can be partial agonists of GABA. Verruculogen increases spontaneous glutamate and aspartate release *in vivo* in guinea pig ileum preparations and also causes an increase in contractile responses to electrical field stimulation, which has been attributed to enhanced release of acetylcholine from presynaptic nerve terminals. Although paxilline blocks high-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels, some related nontremorgenic fungal metabolites, such as paspaline, have also been shown to inhibit these particular ion channels (Burrows and Tyril, 2001). Paxilline also inhibits the cerebellar inositol 1,4,5-triphosphate receptor. Paspalinine and the paspalitrems are thought to impair GABA- and glycine-mediated inhibitory pathways (Burrows and Tyril, 2001).

## TOXICODYNAMICS

### Central effects

Tremorgenic mycotoxins are known to act on the CNS, causing sustained tremors, convulsions and, occasionally, deaths in animals. In general, the clinical signs and symptoms typically observed during tremorgenic mycotoxicoses include diminished activity and immobility, followed by hyperexcitability, muscle tremor, ataxia, tetanic

seizures and convulsions (Cole and Cox, 1981). These clinical signs are reversible if the affected animal is removed from the tremorgen-contaminated source. Death is limited by the emetic effect of these tremorgens. In dogs, the clinical disease involves the development of muscle tremors and seizures, vomiting, alterations in behavior, hyperthermia, depression, coma and pulmonary edema.

Depending on the level of tremorgenic mycotoxin exposure, death can occur in some instances within 2–4 h and is usually secondary to respiratory compromise, metabolic acidosis and/or hyperthermia. In severe cases, clinical signs of toxicosis can persist for several days, and fine tremors can be seen for a week or more. The tremors produced by penitrem A are very similar in most species and begin within a few minutes of intraperitoneal (IP) injection and even sooner with IV injection. The tremors can be sustained and lead to both ataxia and episodic spasms (Cavanagh *et al.*, 1998). Larger doses of penitrem A can cause seizures, massive liver necrosis and death (Hocking *et al.*, 1988). In a histopathological study in rats, Breton *et al.* (1998) revealed that penitrem A induced dose-related injuries in the cerebellum with massive degeneration of Purkinje cells and a significant vacuolization within the molecular layer. In one instance of neurological disorders in dairy cattle associated with *Aspergillus clavatus*-contaminated beer residues, neuronal degeneration was observed within the brainstem and ventral spinal cord, but no analyses for tremorgenic mycotoxins were performed in order to confirm a causal relationship between any tremorgens and the observed pathology (Loretti *et al.*, 2003).

### Peripheral effects

Tremorgenic mycotoxins have also been studied for peripheral effects (McLeay *et al.*, 1999). The tremorgenic mycotoxins, such as penitrem A, paxilline and lolitrem B, can have profound effects on electromyographic (EMG) activity of smooth muscle of the reticulorumen in conscious sheep, with a time course of action similar to their respective characteristic effects on the induction (1–2, 15–20 and 20–30 min) and the duration (1–2, 1–2 and 8–12 h) of tremors. Response to penitrem A revealed a greater sensitivity of smooth muscle than skeletal muscle. The excitatory local effects were partially blocked by atropine, indicating that stimulation of muscarinic cholinergic receptors was involved. Increased local activity may mediate a reflex inhibition of cyclical contractions. However, a nontremorgenic isomer of lolitrem B (31-epi-lolitrem B) had no effect on the reticulorumen. The intensity and duration of the effects of lolitrem B (up to 12 h) indicate that severe disruption of digestion may occur in animals grazing *Neotyphodium lolii*-infected pasture. Moderately severe muscle contractions, traumatic events



secondary to ataxia and prolonged recumbency, especially in large animals, can potentially result in muscle damage following suspected exposure to tremorgenic mycotoxins, but directly myotoxic effects of some mycotoxins cannot be ruled out (Loretta *et al.*, 2003).

### Genotoxicity

Five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen and verrucosidin), which have been associated with molds found in fermented meats, were assessed for genotoxicity (Sabater-Vilar *et al.*, 2003). The mycotoxins were tested in two short-term *in vitro* assays using different genotoxic endpoints, in different phylogenetic systems, with the use of mammalian-microsome assay and the single-cell gel electrophoresis assay of human lymphocytes. The findings revealed that all of the tested mycotoxins, with the exception of penitrem A, exhibited a certain degree of genotoxicity. Verrucosidin appeared to have the highest toxic potential, testing positive in both assays. Verruculogen tested positive in the *Salmonella*/mammalian-microsome assay, and paxilline and fumitremorgen B caused DNA damage in human lymphocytes.

### Acute toxicity

Acute toxicity data are available for only a few of the tremorgenic mycotoxins. It should be noted that the relative doses resulting in lethality in different species might not necessarily be reflective of the relative tremorgenic potentials of these mycotoxins. The LD<sub>50</sub> of penitrem A in mice is 15–19 mg/kg, IP (Ling *et al.*, 1979). The LD<sub>50</sub> of verruculogen is reported to be 15.2 mg/kg, IP, and 266 mg/kg, following oral exposure, in the chicken, with the corresponding LD<sub>50</sub> values in mice being 2.4 mg/kg, IP, and 127 mg/kg, following oral exposure. The LD<sub>50</sub> value of territrems A in mice is 15–19 mg/kg, IP (Ling *et al.*, 1979).

### Potential interactions

In previous studies, concurrent production of roquefortines and penitrem A by various species of *Penicillium* (e.g., *Penicillium crustosum*, *Penicillium cyclopium* Westling and *Penicillium commune*) was demonstrated in culture extracts (Vesonder *et al.*, 1980; Wagener *et al.*, 1980; Kyriakidis *et al.*, 1981; Mantle *et al.*, 1983). Braselton and Rumler (1996) first reported the concurrent presence of both tremorgenic mycotoxins in naturally occurring field cases of canine intoxication and raised the issue of the potential synergistic interactions between these tremorgens. In two recent cases, both Boysen *et al.* (2002)

and Yount *et al.* (2003) diagnosed concurrent intoxication with penitrem A and roquefortines in several dogs. Given that roquefortines and penitrem A can have similar mechanisms of action, there might be the potential for some synergism between these tremorgenic mycotoxins or at least a reduction in the amount of ingested contaminated material required for the onset of clinical signs. Likewise, it would seem logical in instances of “grass staggers” that a greater concentration of multiple, potential tremorgenic mycotoxins in contaminated forages would increase the likelihood and, potentially, the severity of observed intoxications. However, the behavior of mycotoxins, especially those interacting with specific receptors, might not be predictable in mixtures, and the paralytic tremorgen roquefortine C might actually be a reliable biomarker for penitrem A in some tremorgenic syndromes (Tiway *et al.*, 2009).

## CLINICAL ASPECTS OF TREMORGENIC MYCOTOXICOSES

### Diagnosis

Diagnosis of tremorgenic mycotoxin-related intoxication is based on clinical circumstances, a history of exposure to or consumption of moldy foodstuffs, clinical signs of tremors and seizures, and, ideally, detection of tremorgenic mycotoxin(s) in the suspected source material, vomitus, gastrointestinal tract contents, or bile. Detection of molds without detection of tremorgenic mycotoxins suggests the possibility but does not confirm the occurrence of a tremorgenic mycotoxicosis. Differential diagnoses should rule out ethylene glycol, strychnine, metaldehyde, methylxanthines, pyrethroid insecticide, nicotine, organochlorine insecticides, bromethalin, acetylcholinesterase-inhibiting insecticides, other potential neurotoxicants and eclampsia in pregnant animals (Hooser and Talcott, 2006). It needs to be pointed out that in field cases of perennial ryegrass staggers, as well as Dallisgrass and Bahiagrass staggers, the onset of clinical signs is most likely related to the intake of lolitrem B and paspalanine and/or paspalitrems, respectively. The effects of these tremorgenic mycotoxins could potentially be more insidious and longer lasting than those of penitrem A (Burrows and Tyrl, 2001; Hooser and Talcott, 2006). Depending on the laboratory, tremorgenic mycotoxins can generally be quantified using LC-MS/MS (Tiway *et al.*, 2009), HPLC-MS (Rundberget and Wilkins, 2002), GC-MS/MS (Braselton and Rumler, 1996), or TLC (Hooser and Talcott, 2006). Analyses for roquefortines and penitrem A are generally more likely to be available to clinicians than analytical procedures for the other tremorgenic mycotoxins.



Detection of roquefortine C in samples from a tremorgenic syndrome, especially in dogs, where penitrem A is not detected, might serve as a biomarker for exposure to this common tremorgen (Tiwary *et al.*, 2009).

## Treatment

In a small animal setting the minimum initial database should include a complete blood count, serum biochemical profile and assessment of acid–base status and urinalysis (Hooser and Talcott, 2006). Depending on the species, the time period since exposure and the presence and severity of clinical signs, suspected poisoned animals should be stabilized then decontaminated by emesis, gastric lavage, activated charcoal and/or administration of cathartics. Given that intoxications with tremorgenic mycotoxins can be characterized by convulsions or, conversely, severe depression and coma, care should be taken not to induce vomiting when contraindicated by an increased risk for aspiration pneumonia. Especially in companion animals, tremors and convulsions might be controlled by diazepam or methocarbamol, but animals unresponsive to these medications can be treated with barbiturates. The animal should be periodically assessed for metabolic acidosis, hyperthermia, pulmonary edema and aspiration pneumonia. With proper initial and ongoing assessment and in the absence of complications, affected animals should recover relatively uneventfully following removal from the source and appropriate decontamination and other therapeutic procedures. Animals with mild clinical signs or progression of clinical abnormalities following discharge should be reassessed to evaluate the potential for neuronal damage.

## Prevention

Care should be taken to avoid the presence of moldy feedstuffs in areas where animals have unsupervised and unrestricted access, and potentially contaminated materials should be disposed of properly. Trash receptacles should have securely fitting lids, and animals should not be free to roam in areas where discarded foodstuffs or refuse are stored or in the vicinity of compost piles. Livestock should not be fed overtly mold-contaminated forages or concentrates, and care should be taken when feeding food- or beverage-manufacturing byproducts. With respect to “grass staggers,” appropriate stocking rates should be maintained, and management practices should be instituted which ensure that animals have access to young growing plants, especially in pastures dominated by perennial ryegrass, Dallisgrass, Bahiagrass, or Bermudagrass (Burrows and Tyrl, 2001).

## CONCLUSIONS

Tremorgenic mycotoxins containing a tryptophan-derived indole moiety affect many mammalian species, especially sheep, cattle and dogs. Improved analytical techniques are likely to increase the likelihood of detection of “known” or, even, “new” tremorgenic mycotoxins in foodstuffs or biological samples associated with clinical neurological syndromes characterized by tremors and/or convulsions. Roquefortines and penitrem A are produced by several species of *Penicillium*, and dogs exposed to these potent tremorgens exhibit tremors and, potentially, severe convulsive episodes and seizures. Several common forages, especially perennial ryegrass, are susceptible to infection by fungi capable of producing indole–diterpene tremorgenic mycotoxins, such as lolitrems and paspalitrems, which cause “grass staggers” in susceptible species. Although multiple mechanisms are involved in the neurotoxicity of tremorgenic mycotoxins, impaired GABA- and glycine-mediated inhibitory pathways and enhanced excitatory neurotransmitter (i.e., glutamate and aspartate) release appear to play major roles in the onset of clinical signs. Removal of the animal from contaminated foodstuffs usually results in recovery, but severely poisoned animals should be properly assessed; their tremors and/or convulsions treated; and appropriate steps taken to avoid complications, such as aspiration pneumonia. Whenever possible, animals should not have unrestricted and unsupervised access to mold-contaminated foodstuffs.

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# Trichothecenes

Michelle S. Mostrom and Merl F. Raisbeck

## INTRODUCTION

While hundreds of fungal metabolites have been discovered that are potentially toxic in animals, five mycotoxins are generally recognized as an agricultural problem in North America: deoxynivalenol (DON), fumonisin, zearalenone, aflatoxin and ochratoxin. This chapter focuses on DON and the other trichothecenes.

More than 180 trichothecene mycotoxins have been recognized in the past 40 years (Grove, 1988, 2000). These fungal metabolites are a group of sesquiterpenoids characterized by a tetracyclic 12,13-epoxytrichothec-9-ene skeleton and a variable number of acetoxy or hydroxyl group substitutions. The epoxy group at C-12 and C-13 is considered essential for toxicity (Figure 94.1). Trichothecenes can be broadly divided into two groups, macrocyclic and non-macrocyclic trichothecenes, based on the presence of a macrocyclic ring linking C-4 and C-15 with diesters (roridin series) and triesters (verrucarin series). *Fusarium* molds or fungi are the most economically important source of trichothecene mycotoxins. The genus includes many field fungi capable of infecting wheat, corn, barley, oats and forages. *Fusarium* is most common in temperate climates, but contamination of grains is reported worldwide (Placinta *et al.*, 1999; JECFA, 2001; CAST, 2003).

Trichothecenes are potent inhibitors of protein synthesis and are toxic to molds, bacteria, plants and animals. The hallmark clinical sign of trichothecene toxicosis in animals is feed refusal, which has led to speculation that animals may not voluntarily consume enough contaminated ration to cause marked poisoning; however, when the only available feedstuffs are contaminated with trichothecenes, poisoning may result. Clinical signs include emesis, feed refusal and weight loss, immunomodulation, coagulopathy and hemorrhage, and cellular necrosis of mitotically active tissues such as intestinal mucosa, skin, bone marrow, spleen, testis and ovary (CAST, 2003). Trichothecenes have caused lethality in horses (Rodricks and Eppley, 1974), man (Joffe, 1974) and cattle (Hsu *et al.*, 1972), but most recent scientific interest has focused on subclinical syndromes including nutritional impairment (Rotter *et al.*, 1996), loss of production from contaminated feeds and immunosuppression (Bondy and Pestka, 2000; Pestka and Smolinski, 2005).

Some of the confusion about clinical effects of trichothecenes results from the discrepancy between controlled experiments and field studies, and from the variable toxicity of contaminated feedstuffs. Studies

TABLE A

Trichothecene	R1	R2	R3	R4	R5
T-2 toxin	OH	OAc	OAc	H	OCOCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>
HT-2 toxin	OH	OH	OAc	H	OCOCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>
Diacetoxyscirpenol	OH	OAc	OAc	H	H
Neosolaniol	OH	OAc	OAc	H	OH
Calonectrin	OAc	H	OAc	H	H

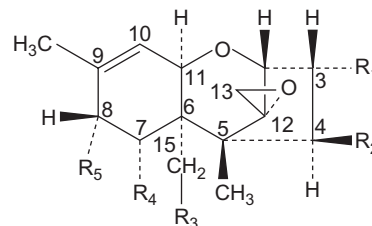


FIGURE 94.1 Chemical structure of Type A trichothecenes. Substitutions R1 through R5 are given above.

using experimental animals and purified trichothecenes often suggest a dose–response relationship for a particular clinical effect that is not supported by field observations. Some naturally contaminated grains are more toxic than can be accounted for by their known mycotoxin content. Controlled experiments using identical methods and similar test animals may yield marked variation, both qualitatively and quantitatively, in response to different batches of naturally contaminated feed. It seems likely that spontaneous trichothecene mycotoxicoses in livestock are complicated by the presence of unidentified mycotoxins or additional fungal metabolites in the ration. Diagnosis may also be frustrated by the difficulty of obtaining a representative feed sample to test and appropriate analytical methodology to identify fungal metabolites.

## BACKGROUND

Mold-infected grains have been associated with ill health in livestock and man for over 100 years. In European Russia and Eastern Siberia, “scabby grains” and “moldy hay” have long been recognized as toxic, and in Japan, a red mold disease or “Akakabi-byo” of wheat caused gastroenteritis in man (Saito and Ohtsubo, 1974). Alimentary toxic aleukia (ATA) of human beings and a similar condition (stachybotryotoxicosis) of horses were associated with overwintered grains and hay during the 1930s and 1940s in the former USSR. Delayed harvest resulted in overwintering of grain, and the cold, wet conditions resulted in the growth of mold on overwintered grain and hay. These molds were later identified as *Fusarium sporotrichioides*, *Fusarium poae* and *Stachybotrys alternans* in grain and hay, respectively, and several trichothecene mycotoxins were isolated. The primary toxin associated with ATA was identified as 4 $\beta$ ,15-diacetoxy-3 $\alpha$ -hydroxy-8 $\alpha$ -[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene or T-2 toxin. A brief description of ATA is informative as it illustrates the classical effects of potent trichothecenes, mainly epithelial irritation or dermonecrosis, gastrointestinal irritation and immunosuppression (Joffe, 1974). Clinically, ATA involved four stages. Stage one occurred shortly after ingestion of toxic grains. Symptoms included a burning sensation of the mouth, tongue, esophagus and stomach and inflammation of the gastrointestinal mucosa accompanied by vomiting, diarrhea, salivation, dizziness and tachycardia. This stage lasted from 3 to 9 days, and coincided with the initial clinical appearance of leukopenia. Stage two was termed the latent or leukopenic stage because the victim felt normal and could function normally, but major changes were occurring in the

hematopoietic system including progressive leukopenia with granulocytopenia, and a relative lymphocytosis. Anemia, icterus and lowered immune resistance to infections were typical of this stage and some reports describe abnormalities in the central and autonomic nervous functions. Stage two lasted from 2 to 8 weeks, with eventual recovery if exposure stopped. If victims continued to consume contaminated grain, the syndrome progressed to a third stage. In stage three, leukopenia worsened and thrombocytopenia and decreased fibrinogen resulted in anemia and petechial hemorrhages on the skin of the trunk, lateral surfaces of the arms, the thighs, face and head. Nasal, gastric and intestinal hemorrhages were noted. Necrotic lesions could appear in the throat, gums, buccal mucosa, larynx and vocal cords with secondary bacterial infections. Lymph node enlargement was observed, and death from stenosis of the glottis was reported. If the victim survived, stage four consisted of a convalescent period of 2 or more months while bone marrow recovered. The clinical signs of ATA in humans have been reproduced in cats given repeated doses of T-2 toxin (Lutsky *et al.*, 1978).

The fungus usually involved in scabby grain blights was identified as *Gibberella zeae* (Schwabe) or *Fusarium graminearum*, its asexual or conidial phase (reviewed by Marasas *et al.*, 1984). A trichothecene, chemically described as 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one, was isolated from barley (Morooka *et al.*, 1972) and field corn infected with *F. graminearum* (Vesonder *et al.*, 1973) and given the trivial name vomitoxin. Vomitoxin, also known as Rd toxin, 4-deoxynivalenol, or DON, was demonstrated to be the *Fusarium* toxin responsible for feed refusal and emesis in monogastric animals fed contaminated corn (Vesonder *et al.*, 1976). Subsequently, an acetylated form of DON, 3-acetoxy-7,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-ADON), was isolated from a *Fusarium* culture that was more toxic than DON in male DDY mice (Yoshizawa and Morooka, 1973).

## SOURCES

Trichothecenes are produced by several genera of fungi, including *Fusarium*, *Stachybotrys*, *Myrothecium*, *Trichothecium*, *Trichoderma*, *Cephalosporium*, *Cylindrocarpon*, *Verticimonosporium* and *Phomopsis* (Scott, 1989). Trichothecene mycotoxins (baccharinoids) have also been isolated from Brazilian plants, notably *Baccharis* spp.; however, all medically and economically important sources to date have been fungal, especially *Fusarium*. *Fusarium* is a major agricultural plant pathogen of temperate growing regions, where it causes *Fusarium*



head blight in wheat, barley, triticale and other grains. *F. graminearum* has an optimum temperature range for growth of 26–28°C at a water activity ( $a_w$ ) greater than 0.88. *Fusarium culmorum* grows optimally at 21°C when  $a_w > 0.87$ . While increased rainfall will increase *Fusarium* head blight, the incidence of blight is primarily affected by moisture at anthesis when the temperature is in the optimum range (Miller, 2002). Moisture at silk emergence and wet weather later in the season increase *Gibberella* or pink ear rot caused by *F. graminearum* in corn.

TABLE B

Trichothecene	R1	R2	R3	R4
Nivalenol	OH	OH	OH	OH
Deoxynivalenol	OH	H	OH	OH
Fusarenon-X	OH	OAc	OH	OH
Diacetylnivalenol	OH	OAc	OAc	OH
3-Acetyldeoxynivalenol	OAc	H	OH	OH
15-Acetyldeoxynivalenol	OH	H	OAc	OH

*Fusarium* species vary in toxigenic potential by strain, which in turn varies with geographic location. Mycotoxin production by *Fusarium* fungi is heavily dependent on oxygen, environmental pH, osmotic tension and sometimes temperature. For example, DON is produced under conditions of low oxygen tension, whereas zearalenone (a non-trichothecene, estrogenic mycotoxin) production by the same fungi requires oxygen saturation, usually occurring after field crops senesce (Miller, 2002). Unusually cool weather conditions in late summer and early fall, coupled with heavy rainfall in the upper Midwest of the United States, can result in widespread, severe *Fusarium* infestation and mycotoxin production. For example, the moldy corn epidemic in Wisconsin during 1962 and 1963 that led to the isolation of toxigenic fungi and the discovery of several important Type A trichothecenes, including T-2 toxin, occurred in such conditions (Bamburg *et al.*, 1968) and again in 1992, climatic conditions produced a bumper crop of various toxigenic *Fusarium*, *F. sporotrichioides*, *F. poae* and *F. graminearum* in Wisconsin corn samples and the isolation of several trichothecenes (DON, T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), neosolaniol and T-2 tetraol) and zearalenone (Park *et al.*, 1996).

Trichothecenes can be chemically classified into four types based on substitutions at five positions of the trichothecene skeleton, including Type A which includes T-2 toxin and HT-2 toxin (Figure 94.1); Type B including nivalenol and DON (Figure 94.2); Type C including crotoxin (Figure 94.3); and Type D or macrocyclics (Figure 94.4). Type A trichothecenes include some of the most toxic trichothecenes, T-2 toxin, its deacetylated metabolite, HT-2 toxin, and DAS (or anguidine). These are most

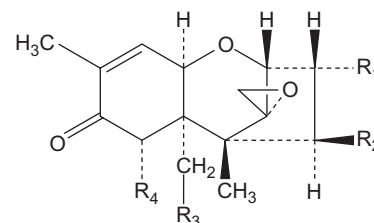


FIGURE 94.2 Chemical structure of Type B trichothecenes. Substitutions R1 through R4 are given above.

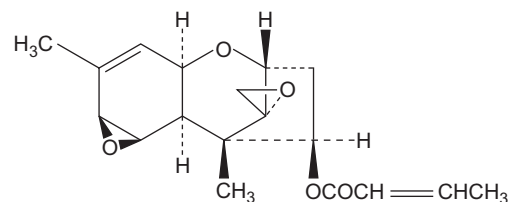


FIGURE 94.3 Chemical structure of Type C trichothecenes (crotoxin).

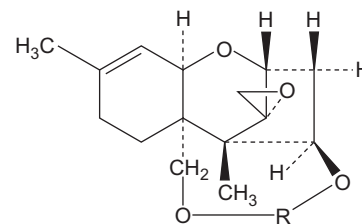


FIGURE 94.4 Chemical structure of Type D trichothecenes and one example substitution.

commonly produced for research in *F. sporotrichioides* and *F. poae* cultures. Both of these *Fusarium* spp. produce toxic metabolites in light or darkness and at low temperatures ( $< -2^{\circ}\text{C}$ ); however, sharp fluctuations in temperature increases the toxicity of extracts. In addition to ATA, moldy grains produced under low temperature conditions have been associated with moldy corn toxicosis (hemorrhagic disease), moldy bean hull intoxication, moldy cerealemesia, fusariotoxicosis and dendrodochiotoxicosis (myrotheciotoxicosis) (Smalley and Strong, 1974).

The Type B trichothecenes are characterized by a keto group at C-8 and hydroxyl group at C-7. These are common natural field contaminants of grains and include DON and its acetylated derivatives, nivalenol (3 $\alpha$ ,4 $\beta$ ,7 $\alpha$ ,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one) and fusarenon-X (4-acetylnivalenol) produced by *F. culmorum* and *F. graminearum*, and other closely related fungi. They are less toxic than the other classes of trichothecenes

without the C-8 keto substitution, such as T-2 toxin, DAS and the macrocyclic trichothecenes.

*F. graminearum* most commonly occurs in southern China, North America and Eastern Europe, and *F. culmorum* more commonly occurs in Western Europe. In North America, *F. graminearum* characteristically produces DON, which can co-occur with another 8-keto-trichothecene, 15-acetyldeoxynivalenol (15-ADON), but in Asia *F. graminearum* almost always produces DON with 3-ADON (Miller *et al.*, 1991). Although not a trichothecene, the estrogenic mycotoxin zearalenone is often found together with DON in North America. Other strains of *F. graminearum*, mostly of Japanese origin, produced nivalenol and fusarenon-X, zearalenone and butenolide. *F. culmorum* strains from the Netherlands produced predominantly DON and 3-ADON, while strains of *F. crookwellense*, collected from four continents, produced 4,15-diacetylnivalenol regardless of the source. More serious adverse effects could be underestimated from moldy corn if only DON, and no acetylated DON or additional trichothecenes, are assayed.

The Type C trichothecenes typically have a second epoxide ring at C-7,8. These trichothecenes are neither produced by *Fusarium*, nor are they associated with adverse effects in livestock. Crotoxin (or antibiotic T) is a Type C trichothecene produced by *Cephalosporium crocinigenum* and *Trichothecium roseum* and has low toxicity in mice (Cole and Cox, 1981). In contrast with Type C trichothecenes, the Type D trichothecenes are potent, cytotoxic compounds with a macrocyclic ring linking C-4 and C-15 on the trichothecene skeleton. The genus *Myrothecium*, including *M. roridum*, *M. leucotrichum* and *M. verrucaria*, can produce verrucarins and roridins that are diesters of the trichothecene verrucarol and acutely toxic to mammalian cell lines *in vitro* and to a wide range of animals with *in vivo* exposure (Mantle, 1991). No consistent naturally occurring toxicoses have been attributed to the *Myrothecium* toxins (Mantle, 1991).

While *Fusarium* does not produce Type D trichothecenes, the fungus *S. alternans* Bonorden (synonyms *S. atra* Corda and *S. chartarum* (Ehrenberg ex Link) Hughes), which grows worldwide on cellulosic vegetation and particularly on mildewed wet straw, produces macrocyclic trichothecene mycotoxins (including satratoxins G and H, verrucarins J and roridin E) that are stable, highly toxic and cause characteristic cytotoxic effects (Bata *et al.*, 1985). Although horses are the most sensitive species, stachybotryotoxicosis has been described in numerous animals, including zoo animals, in many countries of Europe, South Africa and India.

In North America, the trichothecenes produced by *Fusarium* are the major contaminants in cereal crops. Optimal production of T-2 toxin by *F. sporotrichioides* in grains occurs at 6–12°C (as compared with zearalenone production at an optimal 19–20°C). Park *et al.* (1996)

noted that trichothecene concentrations were increased in corn kept in the field and harvested later in cold winter temperatures (grain moistures often greater than 40%), but zearalenone levels were highest in samples collected in the early fall. The co-occurrence of T-2 toxin, DON and zearalenone in field corn also occurs quickly after hail damage in the late summer or fall as hail damage allows fungi to invade the forming seed. Delayed harvests under wet conditions affect other crops. In the United States upper Midwest, a humid, wet fall allowed field molds, such as *Alternaria*, *F. graminearum* and *Phomopsis*, to infect soybeans and produce mycotoxins that include HT-2, DAS, DON and zearalenone (Jacobsen *et al.*, 1995).

In unusually wet, cool periods, *Fusarium* mycotoxins not only occur in the grain, but also in the vegetative part of the plant. *Fusarium* infection of immature corn generally results in higher trichothecene and zearalenone concentrations in the cob, as compared with the mycotoxin concentrations in the kernels. Thus, avoiding incorporation of the cob in animal feed can potentially reduce exposure to mycotoxins. Usually, grain stored at moisture concentrations less than 0.70  $a_w$  or 14.5% moisture by weight is not susceptible to fungal growth or mycotoxin production.

Mycotoxin production occurs in hay, green feed, straw and silage. Hay baled wet or stored in higher moisture conditions (>20%) is subject to molding. Fungal growth in silage usually takes place on the front edges of silage in bunker silos or where silage is not adequately packed and aerobic conditions exist. *Fusarium* occurrence increases with the practice of no-till farming and utilizing corn in crop rotations. Hay, straw, or silage from *Fusarium*-contaminated wheat, barley, oats and corn can contain high levels of trichothecenes, particularly DON and acetylated metabolites. Straw and hay contamination with DON may be as high as 100–150 mg DON/kg (dry weight basis) in wet years (Mostrom *et al.*, 2005).

Of the trichothecenes, DON is probably the most commonly detected in cereal grains throughout the world (Rotter *et al.*, 1996; CAST, 2003). This mycotoxin is resistant to milling and processing and readily enters the animal feed and human food chains. Foodborne trichothecene contamination, in particular DON, has been linked to acute human toxicoses in China, India and Japan, but little information is available regarding potential health effects from chronic exposure (Bhat *et al.*, 1989; Kuiper-Goodman, 1994; JECFA, 2001).

## TOXICOKINETICS

Trichothecenes undergo all four basic reactions in xenobiotic metabolism. Phase I hydrolysis and oxidation and phase II glucuronide conjugation occur in the body

tissues, while reduction of the 12,13-epoxide is thought to occur through microbial action in the gastrointestinal tract; although T-2 toxin is the only trichothecene for which all four basic reactions or pathways occur simultaneously in the same animal (Swanson and Corley, 1989). The ability to remove the epoxide oxygen (deep-oxidation) is an important step in the detoxification of trichothecenes. Orally administered trichothecenes do not accumulate to a significant extent in the body and are rapidly excreted within a few days in urine and feces (bile) (Swanson and Corley, 1989).

Understanding the toxicokinetics of trichothecenes is important for understanding potential effects in animals. Swine are especially sensitive to DON and kinetic parameters have been studied related to intravenous and acute and chronic oral DON exposures (Coppock *et al.*, 1985a; Prelusky *et al.*, 1988, 1990; Prelusky and Trenholm, 1991; Goyarts and Dänicke, 2006). Following an intravenous dose of DON at 1 mg/kg body weight in swine, the mycotoxin distributed rapidly to all tissues and body fluids and declined to negligible levels in all tissues sampled except urine and bile within 24 h (Prelusky and Trenholm, 1991). DON can be detected very rapidly (less than 2.5 min) in the cerebral spinal fluid following intravenous administration in swine; following oral administration of DON in pigs, the plasma DON concentrations correlated closely with cerebral spinal fluid DON levels (Prelusky *et al.*, 1990). Extensive DON tissue accumulation was not detected after dosage indicating that accumulation of edible tissue residues in swine is unlikely at low level DON exposure (Prelusky and Trenholm, 1991). Coppock *et al.* (1985a) reported no detectable residues of DON in skeletal muscle 24 h after intravenous DON administration in pigs. The reported half-life of DON in pigs after an intravenous injection of DON at 0.5 mg/kg body weight ranged between 2.08 and 3.65 h, suggesting that 97% of DON given would be eliminated in 10.1–18.3 h (Coppock *et al.*, 1985a). Following a lower intravenous dose of DON in pigs (0.053 mg/kg body weight), serum DON concentrations decreased biphasically with terminal elimination half-lives ( $t_{1/2\beta}$ ) of between 4.2 and 33.6 h (Goyarts and Dänicke, 2006).

After oral exposure, DON is rapidly and nearly completely absorbed in the stomach and proximal small intestine of pigs (Dänicke *et al.*, 2004a). Pigs dosed with DON at 5.7 mg DON/kg diet chronically for 4 weeks or with one single oral acute exposure (one feeding) had quick absorption of greater than 50% of the DON administered that was highly distributed, with an apparent volume of distribution ( $V_d$ ) higher than total body water, and serum elimination half-lives of 6.3 and 5.3 h in the chronic and acute DON-fed pigs, respectively (Goyarts and Dänicke, 2006). A total of 97% of the DON dose (five elimination half-lives) would be eliminated in 31.5 and 26.5 h after feeding DON chronically or in

one single, acute exposure, respectively. The majority of DON ingested from dietary exposure was eliminated in the urine and feces, with urine being the main excretory route of DON in an unmetabolized form. Similar results were reported by Prelusky *et al.* (1988) who observed that after intravenous DON administration (0.30 mg/kg) in swine, DON was rapidly cleared primarily unchanged in the urine, with minor elimination in bile. The metabolite deepoxy-DON was found in pigs fed the DON-contaminated diet chronically or for a period of longer than 4 weeks (Goyarts and Dänicke, 2006). Eriksen *et al.* (2003) fed pigs a commercial diet containing 3-ADON at 2.5 mg/kg for 2.5 days. Following ingestion, DON was quickly detected in the plasma (indicating deacetylation), with approximately 42% present as a glucuronide conjugate. DON was excreted mainly in urine, with a smaller amount excreted in feces. Deepoxy-DON constituted 52% of the 3-ADON metabolites detected in feces. The deepoxy-DON metabolite increased in concentration from the distal small intestine to the rectum indicating that deepoxidation occurs in the hindgut, which would have minimal influence on toxicity because of major absorbance of DON in the upper gastrointestinal tract.

In contrast with the poor metabolism of DON by swine, the rumen is capable of extensive metabolism of DON and other trichothecenes. The major metabolite of oral DON in ruminants is 3 $\alpha$ ,7 $\alpha$ ,15-trihydroxytrichothec-9, 12-diene-8-one (deepoxy-deoxynivalenol or DOM-1). Deepoxidation of DON to DOM-1 is considered a deactivation step resulting in a non-cytotoxic compound; thus, ruminal metabolism serves a protective function. Because biotransformation occurs in the rumen, little parent compound is available for absorption (Prelusky *et al.*, 1986a; He *et al.*, 1992). Côté *et al.* (1986) reported cows fed high concentrations of DON (66 mg/kg diet for 5 days) excreted approximately 20% of the DON fed in urine and feces as unconjugated DOM-1 (96%) and DON (4%). Preliminary data from the study suggested to the authors that a portion of the remaining 80% of the dose was excreted as glucuronide conjugates of DON and DOM-1 in urine. Dänicke *et al.* (2005) utilized six cannulated dairy cows to evaluate DON and zearalenone metabolism and the effects of *Fusarium*-contaminated wheat on nutrient utilization. The cows were fed a 50:50 mixture of grass silage and contaminated wheat (3.1 mg DON/kg and 0.186 mg zearalenone/kg total ration) providing approximately 25 mg DON/cow/day intake or 0.04 mg DON/kg body weight/day for 4 weeks. The ration was restrictively fed and no feed refusal was observed. Only a small fraction (about 4–28%) of DON was recovered at the duodenum in the form of DON and DOM-1. DOM-1 accounted for 89% of duodenal flow of this mycotoxin. The authors concluded that extensive ruminal metabolism or absorption by rumen mucosa resulted in low DON recovery in

the duodenum. No changes were detected in pH or volatile fatty acid concentrations in rumen fluid from cows fed control wheat versus *Fusarium*-contaminated wheat. In contrast, the ammonia concentrations in rumen fluid were significantly higher from 90 min to 5 h after feeding the *Fusarium*-contaminated wheat, as compared to control wheat. Additionally, the flow of utilizable protein and microbial protein were reduced at the duodenum, suggesting altered ruminal protein utilization could be either the result of mycotoxins in the rumen or from *Fusarium* damage to the structure (cell wall) of wheat grain.

Prelusky *et al.* (1986a) reported that sheep dosed intravenously with DON rapidly excreted two major metabolites of DON as conjugated DON and conjugated DOM-1 in urine, with elimination half-lives of 2.2 and 3.1 h, respectively. Total recovery was 66.5% of the dose, with 63% in the urine and 3.5% in the bile made up of primarily conjugated DOM-1. This suggests the predominant route for elimination in sheep is biotransformation prior to excretion.

Poultry have a greater tolerance to trichothecenes than monogastric mammals because of poor absorption following oral exposure, extensive metabolism and rapid elimination from the body (Prelusky *et al.*, 1986b; Gauvreau, 1991). Oral administration of DON in turkeys (*Meleagris gallopavo*) revealed that 0.96% of the dose was absorbed from the gastrointestinal tract with rapid excretion of DON and its metabolites in urine and excreta (Gauvreau, 1991). The terminal elimination half-life in turkeys following an intravenous dose of DON was about 44 min. Tissue residues of DON and/or metabolites declined rapidly to trace levels.

Lactating cows or laying hens consuming elevated concentrations of DON (>5 mg/kg) transfer minimal concentrations of DON to the milk or eggs, respectively. Charmley *et al.* (1993) fed 0.59, 42 and 104 mg DON/head/day from contaminated corn and wheat for 10 weeks in a lactation study and found no detectable residues (<1 ng/ml) of DON or DOM-1 in milk. Prelusky *et al.* (1984) evaluated the absorption and distribution of a single large oral dose, 920 mg of DON via rumen intubation, given to each of two lactating cows. Maximum blood levels occurred 4.7 and 3.5 h after DON administration and were 200 and 90 ng/ml serum (DON and conjugates, respectively). By 24 h post-dosing, only trace levels (<2 ng DON/ml serum) were detected. Free and conjugated DON were detected in cows' milk at low levels (<4 ng/ml) with an estimated 0.0001% of administered dose excreted in milk. Following a 5-day oral exposure trial to high concentrations of DON (~66 mg/kg in the diet), lactating dairy cows excreted unconjugated DOM-1 in the milk at concentrations up to 26 ng/ml (Côté *et al.*, 1986). No DON was detected (detection limit of 10 µg DON/kg tissue) in eggs or tissues of Leghorn chicks and laying hens and broiler chickens fed a ration containing

4–5 mg DON/kg for 28–160 days (El-Banna *et al.*, 1983). Valenta and Dänicke (2005) did not detect DON, DOM-1, or glucuronide conjugates of these compounds in the yolk or albumen of laying hens fed a maize diet containing 11.9 mg DON/kg dry matter for 16 weeks.

T-2 toxin metabolism has been studied in laboratory and agricultural animals. Metabolism of T-2 toxin generally results in reduced toxicity with deacetylation of T-2 toxin to HT-2 (at C-4), to 4-deacetylneosolaniol, and to T-2 tetraol. T-2 toxin is considered 1.5–1.7 times as toxic as HT-2, which is 4.8 times as toxic as T-2 tetraol (Ueno *et al.*, 1973). Human and bovine liver homogenates are capable of deacetylating T-2 toxin *in vitro* to HT-2 toxin (Ellison and Kotsonis, 1974). T-2 toxin and metabolites in swine can be eliminated as glucuronide conjugates into bile and undergo deconjugation in the intestinal tract by microbial action and enterohepatic recirculation (Corley *et al.*, 1985).

After receiving three daily oral doses of 180 mg T-2 toxin/day (equivalent to dietary levels of 31–35 mg T-2 toxin/kg), a lactating Jersey cow (375 kg) was orally dosed with 156.9 mg of tritium-labeled T-2 toxin (Yoshizawa *et al.*, 1981). The cow showed a good appetite, but milk and urine production decreased by 38 and 50%, respectively, during the experimental period. Plasma concentrations peaked at 8 h after the tritium-T-2 toxin dose, at 64 ppb (ng/g) T-2 toxin equivalents and by 72 h, almost all radioactivity had been eliminated in the urine and feces, in a ratio of 3:7, respectively. About 0.2% of the tritium-T-2 toxin dose was transmitted into the milk with the maximum level of radioactivity in milk at 16 h post-tritium dose of 37 ppb (T-2-toxin equivalents). The cow was killed and tissues were analyzed for T-2 toxin. T-2 toxin was rapidly metabolized and little T-2 toxin accumulated in organ tissues (muscle, liver, kidney, fat, heart, bile, ovaries and mammary gland), but bile and liver contained higher tritium residues than whole blood. The authors considered that a large amount of the absorbed toxin and its metabolites were eliminated via the bile into the intestinal tract. The delayed elimination of large amounts of radioactivity in the feces indicated that T-2 toxin and its metabolites probably recirculate in the enterohepatic system.

A pregnant Holstein cow was intubated with 182 mg of purified T-2 toxin daily (equivalent to about 0.5 mg T-2 toxin/kg body weight) for 15 consecutive days (Robison *et al.*, 1979). Milk samples collected on days 2, 5, 10 and 12 contained T-2 toxin ranging from 10 to 160 ng/g. The dose given to this cow corresponds to an unusually high feed concentration of 50 mg T-2 toxin/kg; therefore, the authors considered it unlikely that T-2 toxin would be detectable in milk at T-2 toxin concentrations found naturally in feeds. The authors fed a sow (170 kg) T-2 toxin at 12 mg/kg diet (equivalent to 0.5 mg T-2 toxin/kg body weight/day) for 220 days. Six days post-parturition a



milk sample was analyzed for T-2 toxin and contained 76 ng/g. Glávits and Ványi (1995) described a "perinatal form of T-2 toxicosis" in swine in Hungary. The authors reported T-2 toxin was excreted in the milk of sows causing lesions, characteristic of T-2, in organs of suckling pigs, including degeneration and necrosis of cells in the bone marrow, and death.

## MICROBIAL METABOLISM

In the ruminant, significant metabolism of trichothecenes occurs in the rumen and gastrointestinal tract prior to absorption. King *et al.* (1984) noted almost complete transformation of DON to a deepoxidation product when DON was incubated *in vitro* with rumen fluid for a 24 h period. Swanson *et al.* (1987) reported DON was partially converted to deepoxidated DON (DOM-1) by rumen microbes. Rumen microbes, in particular protozoa, appear to be active in the deacetylation of the trichothecenes T-2 toxin and DAS to HT-2 and monoacetoxyscirpenol (Kiessling *et al.*, 1984). The authors reported no effect of ovine rumen fluid on DON metabolism; however, the incubation only lasted 3 h. Several rumen bacteria with esterase activity (*Butyrivibrio fibrisolvens*, *Selenomonas ruminantium* and *Anaerobivrio lipolytica*) degraded T-2 toxin to HT-2 toxin and T-2 triol, but *B. fibrisolvens* also produced neosolaniol (Westlake *et al.*, 1987a,b). The authors suggested that at least two different *B. fibrisolvens* enzymes, differing in their specificity for side chains, were responsible for T-2 toxin degradation.

Microbes in the large intestines of chickens are capable of complete DON transformation *in vitro* to a deepoxy metabolite, whereas no metabolism of DON was reported with the *in vitro* incubation of swine large intestinal contents (He *et al.*, 1992). In contrast, Hedman and Pettersson (1997) reported that five of six pigs (weighing about 18 kg) fed nivalenol at 2.5 or 5.0 mg/kg in the diet for 1 week excreted a deepoxidated nivalenol in feces. After 3 weeks on the nivalenol diet, the sixth pig acquired the ability to deepoxidate nivalenol. Additionally, DON was deepoxidated during *in vitro* incubation with microbes that had deepoxidated nivalenol. No deepoxidated nivalenol was detected in feces after feeding a diet of 2.5 or 5 mg nivalenol/kg diet to broiler chickens for 3 weeks, although an unidentified metabolite was found (Hedman and Pettersson, 1997). The ability of microbes to acquire the capability to deepoxidate or detoxify trichothecenes may account for the time course of clinical signs associated with trichothecene ingestion. Microbial adaptation may require a period of several weeks during which the host animal

is exposed to more of the toxic parent trichothecene. The rate and location of deepoxidation of trichothecenes prior to absorption is important in the development of toxic effects. Formation of deepoxides higher in the gastrointestinal tract would reduce the potential toxicity of the trichothecene. Biotransformation of DON is inhibited by low pH *in vitro*, with a pH of 5.2 completely inhibiting DON metabolism either by inactivating the microorganisms or specifically inhibiting the deepoxidation process of DON (He *et al.*, 1992). This may have major implications in the metabolism of DON by ruminants on higher grain diets causing production of organic acids that reduce the rumen pH. As can be seen by the aforementioned, the apparent resistance of ruminants to trichothecenes is due to ruminal metabolism.

## MECHANISM OF ACTION AND TOXICITY

Trichothecenes have multiple effects on eukaryotic cells, including inhibition of protein, RNA and DNA synthesis, alteration of membrane structure and mitochondrial function, stimulation of lipid peroxidation, induction of programmed cell death or apoptosis, and activation of cytokines and chemokines (Table 94.1). It is believed that the primary effect of trichothecenes is inhibition of protein synthesis as all of the other reported effects might be secondary to decreased protein synthesis (Rocha *et al.*, 2005).

TABLE 94.1 Trichothecene effects on eukaryotic cell functions

Inhibit protein synthesis	Ehrlich and Daigle (1987), McLaughlin <i>et al.</i> (1977)
Inhibit RNA and DNA synthesis	Rosenstein and Lafarge-Frayssinet (1983), Thompson and Wannemacher (1986)
Stimulate lipid peroxidation	Rizzo <i>et al.</i> (1994), Vila <i>et al.</i> (2002)
Alter cellular membrane function	Bunner and Morris (1988)
Inhibit mitochondrial and electrontransport chain function	Pace <i>et al.</i> (1988)
Induce apoptosis	Pestka <i>et al.</i> (1994), Shinozuka <i>et al.</i> (1998), Islam <i>et al.</i> (1998)
Activate MAPKs	Zhou <i>et al.</i> (2005)
Modulate immune responses	Corrier (1991), Bondy and Pestka (2000)
Alter neurotransmitters	Prelusky <i>et al.</i> (1992), Swamy <i>et al.</i> (2004)
Induce gene expression of numerous chemokines and cytokines	Azcona-Olivera <i>et al.</i> (1995), Zhou <i>et al.</i> (1997), Moon and Pestka (2002), Ji <i>et al.</i> (1998), Pestka <i>et al.</i> (2005)

## PROTEIN SYNTHESIS INHIBITION

Trichothecenes bind to ribosomes in eukaryotic cells, in particular to the 60S ribosomal subunits, and interfere with peptidyl transferase activity (McLaughlin *et al.*, 1977). Inhibition of protein synthesis requires an intact 9,10 double bond and the C-12,13 epoxide. Trichothecenes can be divided into two groups based on their site of action on protein synthesis, either preferential inhibition of initiation or inhibition of elongation or termination. Trichothecenes with hydroxyl and acetyl substitutions at both C-3 and C-4, such as T-2 toxin, DAS, scirpentriol and verrucaric acid, predominantly inhibit initiation, and compounds such as trichodermin, crotoxin, crotoxin and verrucarol inhibit elongation or termination (McLaughlin *et al.*, 1977). The cytotoxicity of DON, a trichothecene with a keto group at C-8 and a hydroxyl group at C-7, results from protein synthesis inhibition at the ribosomal level during the elongation and termination step in mammalian cells (Ueno, 1983; Ehrlich and Daigle, 1987). In an *in vivo* study, Robbana-Barnat *et al.* (1988) dosed Balb-c mice with DON by intraperitoneal injection (4, 10, 20 and 80 mg DON/kg body weight) followed by an injection of radio-labeled leucine, killed the mice 5 h post-dosage, and determined leucine incorporation into proteins. Mice dosed intraperitoneally at 10 mg DON/kg body weight or more had marked inhibition of protein synthesis in cardiac tissue. Mice fed a diet with 10 mg DON/kg ration for several weeks exhibited cardiac lesions, primarily foci of calcified pericarditis, which suggested a preferential effect on cardiac tissue in the mice. In an *in vivo* low dose DON study in pigs, protein synthesis (using the "flooding dose technique" with radio-labeled phenylalanine as tracer and expressed as fractional synthesis rate) was significantly reduced in kidneys, spleen and ileum of pigs exposed orally to 5.7 mg DON/kg diet for about 4 weeks (Dänicke *et al.*, 2006). Protein synthesis of the liver, skeletal and cardiac muscle, mesenteric lymph nodes, duodenum, jejunum, pancreas and lung was not significantly affected by oral DON exposure.

Trichothecenes inhibit both RNA and DNA synthesis. Using hepatoma cells and phytohemagglutinin-stimulated lymphocytes in an *in vitro* culture, Rosenstein and Lafarge-Frayssinet (1983) reported T-2 toxin inhibited DNA synthesis. DON was demonstrated to inhibit DNA synthesis in splenic lymphocytes and human peripheral blood lymphocytes (Mekhancha-Dahel *et al.*, 1990). Thompson and Wannemacher (1986) reported trichothecenes strongly inhibited RNA synthesis in HeLa cells and had only slight inhibitory effects on Vero cells. The inhibition of nucleic acid synthesis is generally considered secondary to protein synthesis. T-2 toxin inhibited mitochondrial protein synthesis and electron

transport action in rat liver cells and *in vivo*, although high doses were used in the studies (Pace, 1983; Pace *et al.*, 1988).

## LIPID PEROXIDATION

T-2 toxin is thought to increase production of oxygen radicals, overwhelming the scavenging system for oxygen radicals and resulting in cell injury. Rizzo *et al.* (1994) administered a single oral dose of DON or T-2 toxin at 28 or 3.6 mg/kg body weight, respectively, to male Wistar rats on antioxidant-deficient diets. Liver peroxides, as measured by thiobarbituric acid-reactive substances, increased 21 and 268% in rats administered DON or T-2 toxin, respectively. Significant decreases in hepatic glutathione concentration and superoxide dismutase activity occurred in the treated rats, as compared with the controls.

Vila *et al.* (2002) studied the effects of T-2 toxin, given by oral gavage at dosages of 4 and 6.25 mg/kg body weight, on vitamin E status and lipid peroxidation in female CD-1 mice. Lipid peroxidation was evaluated by the plasma and organ content of malondialdehyde (MDA). Mice had a decrease in plasma vitamin E concentration 16 h after toxin treatment, and the concentration remained significantly depressed for up to 48–72 h. The MDA content of liver increased significantly 24–48 h after T-2 toxin administration, as compared with control mice, and returned to the control range after 72 h. These findings suggest that T-2 toxin results in lipid peroxidation in mice and vitamin E is consumed by scavenging free radicals from damage by T-2 toxin.

## NEUROTRANSMITTER EFFECTS

DON produced two main clinical signs in monogastrics, vomiting, or emesis at higher concentrations and reduced food consumption or feed refusal at lower concentrations (Forsyth *et al.*, 1977). The exact mechanism(s) of action of DON which result(s) in feed refusal is/are not known. DON could act directly via a central mechanism controlling hunger or satiety or possibly peripherally through non-specific mechanisms inducing lethargy or depression. Prelusky *et al.* (1992) evaluated the effects of DON on brain biogenic amine concentrations after administration of a single intravenous dose of 0.25 mg DON/kg body weight to young swine (15–23 kg body weight). Norepinephrine was significantly increased and dopamine significantly decreased

in the brain regions of the hypothalamus, frontal cortex and cerebellum for up to 8 h post-treatment, as compared with control animals. Serotonin or 5-hydroxytryptamine was initially increased 1 h post-treatment in the hypothalamus and by 8 h post-treatment was significantly decreased in the hypothalamus and frontal cortex, as compared with controls. The authors concluded that changes in neurotransmitters were not consistent with known neurochemical changes associated with chemically induced anorexia, but might be due to other toxicological responses, such as vomiting. Neurotransmitter concentrations were measured in the cortex, hypothalamus and pons of starter pigs (9.3 kg) fed 21 days of control and *Fusarium*-contaminated feeds (approximately 3.9 and 5.8 mg DON/kg diet) and in 1-day-old male broiler chicks fed *Fusarium*-contaminated feeds (approximately 6.3 and 9.7 mg DON/kg diet) for 56 days (Swamy *et al.* 2004). The pigs on mycotoxin-contaminated feeds had alterations in regional brain neurochemistry, including a linear increase in cortex serotonin concentrations with a linear decrease in hypothalamic tryptophan and norepinephrine concentrations and pons dopamine concentrations. At the end of the broiler study, chicks on mycotoxin-contaminated diets had linear increases of serotonin in the pons and cortex, and norepinephrine and dopamine concentrations in the pons. Perhaps through peripheral action on serotonin-3 receptors, rodents displayed inhibited gastric emptying in a dose-related manner when DON was given orally at 50–1000 µg/kg body weight (Fioramonti *et al.*, 1993). Intestinal propulsion was reduced at the highest dose (1000 µg/kg). This effect is not common to all trichothecenes because T-2 toxin accelerated gastric emptying under similar experimental conditions (Fioramonti *et al.*, 1987).

While the exact role(s) of these neurotransmitters in eating disorders is/are not known, Swamy *et al.* (2004) suggested that stimulation of serotonergic and  $\alpha_2$ -noradrenergic receptors in the hypothalamus of the brain was important in feeding behavior. The increase in serotonergic neurotransmitter concentrations and decrease in norepinephrine concentrations in the brains of pigs fed mycotoxin-contaminated diets might explain some aspects of feed refusal.

Borison and Goodheart (1989) evaluated neural factors involved in the acute emetic, cardiovascular and respiratory effects of T-2 toxin injected (intravenously or intraperitoneally) at 2 mg/kg body weight in cats. While the causation of vomiting has been attributed to the stimulation of the chemoreceptor trigger zone in the area postrema of the medulla oblongata, area postrema-ablated cats exhibited vomiting 5 h after T-2 administration, as compared with normal cats that vomited within an hour of T-2 administration, and generally persisted in vomiting until the time of death at about 12 h. The authors

concluded that the area postrema was not the sole receptor site of trichothecene action to induce emesis. They hypothesized that a possible mechanism of delayed vomiting from trichothecenes may be similar to that of acute radiation sickness in cats exhibiting emesis and malaise, which is mediated by neural afferent pathways from the abdomen that traverse the vagus nerve and dorsal columns of the spinal cord.

## IMMUNOTOXICITY

The molecular basis for cytotoxic effects of trichothecenes and immunosuppression is directly or indirectly related to inhibition of protein synthesis. The most potent immunosuppressive trichothecenes are T-2 toxin, DAS, DON and fusarenon-X, which are the most potent protein synthesis inhibitors (Corrier, 1991). In addition, the occurrence of other secondary metabolites from *Fusarium* associated with trichothecene biosynthesis, such as substituted neosolaniols and calonecetrins, inhibit T- and B-lymphocytes when evaluated in mouse spleen lymphocyte proliferation assays (Bondy *et al.*, 1991). The most widely studied trichothecene, because of potential acute toxicity and use as a chemical warfare agent, is T-2 toxin. T-2 toxin caused necrosis and lymphoid depletion in the thymus, spleen and lymph nodes of a variety of laboratory animals (Ueno, 1977; Hayes *et al.*, 1980; Taylor *et al.*, 1989), swine (Weaver *et al.*, 1978a), cattle (Osweiler *et al.*, 1981) and sheep (Friend *et al.*, 1983). Necrosis and thymic depletion have been reported in the thymus, bursa of Fabricius and spleen in chickens treated with T-2 toxin (Hoerr *et al.*, 1981). Age of exposure is important, as neonatal animals are more sensitive than older animals. Holladay *et al.* (1993) dosed pregnant mice by oral gavage with 1.2 or 1.5 mg T-2 toxin/kg body weight on gestational days 14–17 and found that lymphocyte progenitor cells appear to be more sensitive than thymocytes to T-2 toxin, and are responsible for fetal thymic atrophy and potential immunosuppression seen with T-2.

Trichothecenes have been shown to both stimulate and impair humoral immunity, cell-mediated immunity and host resistance in experimental and food animals (reviewed by Corrier, 1991; Pestka and Bondy, 1994; Rotter *et al.*, 1996; Bondy and Pestka, 2000; Pestka and Smolinski, 2005). The dose and duration of toxin exposure in relation to the timing of the immune assay determined whether stimulation or suppression resulted. For example, Bottex *et al.* (1990) reported contradictory effects in Swiss mice given intraperitoneal doses of 5.3 or 2.7 mg DAS/kg either prior to (range of –10 to –3 days) or following (range of +3 h to +5 days) intraperitoneal injection of *Salmonella typhimurium*. Mice injected with



DAS prior to *Salmonella* infection had a lower mortality rate than control mice given just *Salmonella*. In contrast, mice injected with DAS following infection had a significantly higher mortality than controls given *Salmonella*. Similar results were observed in mice given intraperitoneal injections of DAS either prior to or following antigenic stimulation with sheep erythrocytes. The splenic plaque-forming cell response against sheep erythrocytes was increased in mice given DAS prior to the antigenic stimulation and significantly inhibited in mice given DAS after antigenic stimulation.

Immunostimulation after small doses of trichothecenes apparently results from induction of immune- and inflammation-associated genes. Trichothecene doses that partially inhibited translation up-regulated expression of immune-related genes including proinflammatory cytokines and chemokines, cyclooxygenase 2 and inducible nitric oxide synthase (Azcona-Olivera *et al.*, 1995; Zhou *et al.*, 1997; Ji *et al.*, 1998; Moon and Pestka, 2002; Pestka *et al.*, 2005). DON regulated interleukin (IL) 2, a cytokine considered to be a central growth and death factor for antigen-activated T-cells, and IL-8, a proinflammatory chemokine that affects host-defense induction of trafficking neutrophils across vascular walls (Pestka *et al.*, 2005).

The frequent occurrence of DON in cereals and potential chronic exposures to contaminated feed sources has focused attention on DON immunomodulation in animals, particularly the impact on susceptibility to infections and altered vaccination responses. Tryphonas *et al.* (1986) suggested a no-effect level in Swiss-Webster mice of 0.25–0.50 mg DON/kg diet/day based on *Listeria monocytogenes* challenge, serum  $\alpha_2$ - and  $\beta$ -globulins, and splenic lymphocyte proliferation to phytohemagglutinin P. Rotter *et al.* (1994) reported no significant differences in lymphocyte proliferation responses to phytohemagglutinin or pokeweed mitogens between treatments of pigs fed 0, 0.95, 1.78 and 2.85 mg DON/kg diet. The pigs responded well to primary immunization with sheep erythrocytes. However, pigs fed higher concentrations of 1.78 and 2.85 mg DON/kg diet, and paired control pigs developed the maximum titer 7 days later than controls. As the level of DON contamination increased, pigs showed increased albumin concentrations, decreased  $\alpha$ -globulin concentrations and increases in the albumin/globulin ratios. Similarly, Accensi *et al.* (2006) observed no significant changes in lymphocyte proliferation to mitogens or in cytokine production of IL-4 and interferon- $\gamma$  in young swine fed low doses of DON (0, 0.28, 0.56 and 0.84 mg/kg diet) for 4 weeks. Øvernes *et al.* (1997) reported a reduction in secondary antibody responses to tetanus toxoid injections in pigs fed 1.8 and 4.7 mg DON/kg feed for about 9 weeks, but no titer reductions to sheep erythrocytes, human serum albumin, paratuberculosis vaccine, or diphtheria toxoid. Peripheral blood lymphocyte responses to

phytohemagglutinin were increased in pigs fed 4.7 mg DON/kg feed for 9 weeks.

In a high dose exposure of poultry to DON, Harvey *et al.* (1991) reported a reduction in immune responses to Newcastle vaccination in Leghorn hens fed a ration of naturally DON-contaminated wheat at 18 mg DON/kg feed for 18 weeks. DON fed at increasing concentrations (5.8–13.6 mg DON/kg feed) to day-old turkey poults for 12 weeks reduced contact sensitivity responses to dinitrochlorobenzene (a CD8<sup>+</sup> T-lymphocyte cell-mediated response), but DON-fed turkeys generated primary and secondary antibody responses to sheep erythrocytes (a CD4<sup>+</sup> T-lymphocyte humoral response) similar to the control group, which was exposed to very low concentrations of DON in the feed (Chowdhury *et al.*, 2005a). In a similar experiment with day-old Pekin male ducklings fed a naturally contaminated ration with DON (6.3–18.6 mg DON/kg feed) for 6 weeks, the cell-mediated response to dinitrochlorobenzene was decreased with no effect on the antibody response to sheep erythrocytes (Chowdhury *et al.*, 2005b). Consumption of contaminated grains by both turkey poults and ducklings resulted in only minor impacts on serum chemistries and hematology, and no effect on body weight gains or feed consumption in the duckling study.

While T-2 toxin exposure in numerous laboratory animal models and other species causes immunosuppression, the specific functions of various cell type affected by the mycotoxin have not been definitively ascertained (Taylor *et al.*, 1989). T-2 toxin given by intraperitoneal administration to mice reduced thymus weights, inhibited antibody synthesis against sheep erythrocytes and prolonged skin graft rejection times (Rosenstein *et al.*, 1979). Rabbits given 0.5 mg T-2 toxin/kg body weight *per os* for 17 or 28 days had reduced antibody responses to aerosolized *Aspergillus fumigatus* challenge (Niyo *et al.*, 1988). Calves dosed orally with T-2 toxin at 0.6 mg/kg/day for 43 days had depressed mitogen responsiveness to phytohemagglutinin at 1, 8 and 29 days of toxin administration and decreased mitogen responsiveness to concanavalin A and pokeweed mitogens on day 29 of toxin administration (Buening *et al.*, 1982). Phagocytosis of 2-hydroxymethacrylate particles by neutrophils was not altered in pigs fed T-2 toxin for 21 days (Rafai *et al.*, 1995b). The authors reported humoral immunity, as measured by titers against horse globulin, a T-dependent antigen, was significantly decreased in pigs fed 14–21 days of T-2 toxin at 0.5, 1, 2 and 3 mg/kg feed. Additional evidence of impaired T-lymphocyte function was found in the reduction of mitogen-induced lymphocyte proliferation tests with phytohemagglutinin, concanavalin A and purified horse IgG at 2 and 3 mg T-2 toxin/kg feed. The authors remarked on minor histological changes including a decrease in the size of thymic lobules and lobular cortex of the thymus, a decrease in white



pulp of the spleen and regressive changes (interpreted as depletion of lymphocytes) of the germinal centers of lymph nodes of T-2 treated pigs.

Humoral immune responses to anaplasma vaccine were not different between T-2-treated and control calves, but calves dosed with 0.6 mg T-2 toxin/kg body weight had significantly lower concentrations of  $\alpha$ -,  $\gamma$ -,  $\beta_1$ - and  $\beta_2$ -serum protein fractions and elevated albumin/globulin ratio compared with control calves (Osweiler *et al.*, 1981). Serum IgA and IgM concentrations were significantly lower for the high dose T-2 toxin calves, as compared with controls. Pathological changes included decreases in body and thymus weights of T-2 toxin-treated calves. Histologically, thymii from calves dosed with 0.3 mg T-2/kg body weight exhibited cortical thinning with loss of mature lymphocytes and fibrous connective tissue separating individual thymic lobules. The high dose calves had almost complete replacement of normal thymic architecture with fibrous connective tissue and loss of the normal lobular pattern and mature lymphocytes. Calves (158–225 kg) dosed orally with T-2 toxin at 0.5 mg/kg body weight for 28 days had reduced serum IgG, IgM, albumin, globulin and complement proteins (Mann *et al.*, 1983). Lambs treated orally with a high dose of T-2 toxin (0.6 mg T-2 toxin/kg body weight) daily were leukopenic on day 7 and lymphopenic on days 7 and 14 (Friend *et al.*, 1983). On day 7 of this study, lambs showed decreased lymphocyte blastogenic responses to concanavalin A and lipopolysaccharide. Mitogen responses improved by day 21 of T-2 exposure. At necropsy, no differences in parasite loads were noted among T-2 treated lambs, suggesting that the dosage of T-2 toxin did not reduce immunity to a threshold allowing a fulminating coccidiosis to occur in treated lambs. Lambs showed no significant alteration of peripheral blood lymphocyte blastogenesis to phytohemagglutinin after 34 days of DAS treatment (5 mg/kg diet), as compared with control lambs (Harvey *et al.*, 1995).

## IgA

Increased serum IgA and potential IgA dysregulation and nephropathy have been reported in rodents after high (about 25 mg/kg) dietary DON exposure. Forsell *et al.* (1986) reported dose-dependent increases in serum IgA and decreases in serum IgM in female B6C3F1 mice fed 0.5, 2, 5, 10 and 25 mg DON/kg diet for 8 weeks. Further investigation with B6C3F1 mice showed that feeding 25 mg DON/kg diet for 4 weeks increased serum IgA, and continued DON feeding for 24 weeks increased serum IgA 17-fold over controls (Pestka *et al.*, 1989). Mice exhibited a shift from primarily monomeric IgA to polymeric IgA and increased *in vitro* splenocyte production of IgA from both spontaneous- and

lipopolysaccharide-stimulated cultures. The DON-associated increase in serum IgA resulted from stimulation of CD4<sup>+</sup> and IgA<sup>+</sup> lymphocytes in Peyer's patches and in the spleen (Pestka *et al.*, 1989; Dong *et al.*, 1991). DON fed mice showed marked mesangial IgA accumulation based on immunofluorescence staining of kidney tissue (Pestka *et al.*, 1989). In humans, deposition of IgA in the mesangium and capillary loops of the kidney can lead to a focal, non-progressive glomerulonephritis (Jones *et al.*, 1997).

Elevated serum IgA in animals exposed to oral DON does not appear to be consistent across species. Elevations in serum IgA concentrations were not observed in horses fed DON-contaminated feed (exposure of ~0.06–0.1 mg DON/kg body weight/day) (Johnson *et al.*, 1997; Raymond *et al.*, 2005). Bergsjø *et al.* (1992) did not detect any significant differences in serum IgA concentrations in pigs fed DON-contaminated diets from 0.5 to 4 mg/kg feed for 100 days. The authors stated that variation between individuals in the group and the low number of pigs per group ( $n = 8$ ) might not have revealed small differences. Weaned pigs fed low concentrations of DON (<0.84 mg DON/kg diet) for 4 weeks (Accensi *et al.*, 2006) or fed higher concentrations of DON (at 5.8 mg/kg) for 3 weeks (Swamy *et al.*, 2003) showed no consistent changes in serum IgA. In contrast, Goyarts *et al.* (2005) found that finishing pigs fed DON-contaminated wheat (6.5 mg DON/kg diet) *ad libitum* had a significant increase in serum IgA concentrations, with male pigs significantly higher than females. Significant differences in serum IgA concentrations were not observed in control and DON-fed pigs on the restricted feeding regimen (assured similar feed intake at the lowest level for all animals of both control and DON groups).

## APOPTOSIS

Apoptosis, a form of programmed cell death, has been proposed to explain the loss of lymphocytes and hematopoietic cells during trichothecene poisoning (Pestka *et al.*, 1994; Shinozuka *et al.*, 1998). Apoptosis normally serves as a self-regulating pathway in the immune system that reduces excessive inflammation and prevents autoimmune disease (Dong *et al.*, 2002); however, inappropriate activation by trichothecenes results in dysfunction. Activation of mitogen-activated protein kinases (MAPKs) by satratoxins and other trichothecenes correlated with and preceded apoptosis (Yang *et al.*, 2000). The authors used two myeloid models, RAW 264.7 murine macrophages and U937 human leukemic cells, in a cleavage assay and determined the potency of cytotoxicity to be satratoxin G, roridin A, verrucarins A > T-2

toxin, satratoxin F, H>nivalenol and vomitoxin. Using flow cytometry cell cycle analysis and phenotypic staining to study *in vitro* effects, Pestka *et al.* (1994) demonstrated that DON could either inhibit or enhance apoptosis in T-, B- and IgA<sup>+</sup> cells from murine spleen and Peyer's patch. Apoptosis was dependent on lymphocyte subset, source of tissue and glucocorticoid induction. Islam *et al.* (1998) demonstrated *in vivo* that T-2 toxin, given by intraperitoneal injection to mice, induced thymic atrophy, DNA fragmentation and histopathological changes in thymic tissue characteristic of apoptosis, including cell shrinkage and nuclear condensation. Macrophages appeared to be very sensitive to trichothecenes and could undergo trichothecene-stimulated apoptosis (Zhou *et al.*, 2005). The authors suggested that in addition to trichothecenes binding to the 60S ribosomal subunits and producing translational inhibition, another molecular pathway for trichothecene induction of apoptosis was through triggering a ribotoxic stress response activating MAPKs. MAPKs modulate cell physiological processes, such as cell growth, differentiation and apoptosis, and are important for signal transduction in immune responses (Dong *et al.*, 2002). For example, T-2 toxin given orally to pregnant rats on day 13 of gestation induced apoptosis in neuroepithelial cells of the fetal rat brain that coincided with expression of oxidative stress-related genes (such as heat shock protein 70 and heme oxygenase) and subsequent activation of MAPKs and caspase-2, which are important factors in cell signaling pathways for apoptosis (Sehata *et al.*, 2004).

## CELL MEMBRANE FUNCTION

At low concentrations (0.4pg/ml to 4ng/ml), T-2 toxin altered several cell membrane functions in L-6 myoblasts, including uptake of calcium, rubidium and glucose, incorporation of thymidine or leucine and tyrosine into DNA or protein, and residual cellular lactate dehydrogenase (Bunner and Morris, 1988). Changes occurred within 10 min of exposure, suggesting to the authors that T-2 toxin directly or indirectly affected glucose, nucleotide and amino acid transporters and calcium/potassium channel activities independent of protein synthesis inhibition.

## REPRODUCTIVE TOXICITY

No studies have indicated that DON is a reproductive toxin at concentrations typical of naturally contaminated feed. Friend *et al.* (1986) reported significant weight

reductions in young male and female pigs fed DON-contaminated feeds at 3.7 and 4.2mg DON/kg (~0.14 and 0.17mg/kg body weight/day) feed for 7 weeks; however, no significant histological changes were observed in the testis (seminiferous epithelium) or ovary (follicle). In contrast, several studies indicate that T-2 toxin can affect reproduction. Glávits *et al.* (1983) reported in a field case involving a large swine herd infertility in gilts and sows that coincided with the detection of T-2 and HT-2 toxins at 1–2mg/kg feed. Pathology revealed cystic degeneration of the ovaries and uterine atrophy. Huszenicza *et al.* (2000) evaluated low oral T-2 toxin exposures of 0, 0.3 or 0.9mg T-2 toxin/day and 9mg T-2 toxin/day for 3 weeks in ewes and heifers, respectively, on a rich, acidosis-inducing concentrate diet. The results suggested that, in ewes and heifers, rumen acidosis along with exposure to low oral T-2 toxin intake might delay maturation of the dominant ovarian follicle and ovulation and shorten corpora lutea lifespan (lower plasma progesterone concentrations); although the number of animals in the experiment was small. In an equine study of trotter mares, the horses were given 7mg purified T-2 toxin/day in oats (~0.01mg/kg body weight/day) for 32–40 days beginning on estrous cycle day 10 (Juhász *et al.*, 1997). Skin lesions were noted around the mouth of three horses; however, no adverse effects were noted on the length of the interovulatory interval, luteal and follicular phases of the estrous cycle, plasma progesterone profiles, or follicular kinetics. Uterine flushing of five mares in the trial yielded three embryos suggesting that T-2 toxin had no detrimental effect on ovarian activity, fertilization, or oviductal transport.

In pregnant rats, T-2 toxin crosses the placenta and is distributed to fetal tissues (Lafarge-Frayssinet *et al.*, 1990). Rousseaux and Schiefer (1987) reported that T-2 toxin caused fetal death at high doses (associated with maternal toxicity), with fetal toxicity primarily in the central nervous system and skeletal system. T-2 toxin administered intravenously at approximately one-third or one-sixth of the LD<sub>50</sub> (0.41 or 0.21mg/kg body weight) to sows at the beginning of the third trimester of pregnancy caused vomiting 90 min post-injection with the sows becoming listless and aborting their litters 48–80 h later (Weaver *et al.*, 1978b). In another study, three sows were fed purified T-2 toxin in a standard swine ration at 12mg/kg diet for up to 220 days, causing clinical signs of repeat breeding, small litters (four piglets) and small (0.37–0.65kg) piglets, which had no gross or histological lesions attributable to T-2 toxin (Weaver *et al.*, 1978a). The sows did not develop changes in the complete blood count, total protein, or alterations in the bone marrow. These studies used concentrations of T-2 higher than commonly found in feeds screened for visible molds.

Thirty 3-week-old Single Comb White Leghorn hens were fed either purified T-2 toxin or DAS at 2mg/kg diet for 24 days (Diaz *et al.*, 1994). Egg production dropped

about 7% in hens fed either T-2 or DAS on days 13–18 of the study, but recovered to near normal by day 24. Shlosberg *et al.* (1984) reported acute, severe reduction in egg production, feed refusal, depression and recumbency, cyanotic appearance of the comb and wattles, and some blue-green discoloration of droppings in a flock of 8-month-old laying hens. Following delivery of new feed, the mean daily egg production dropped from about 2400 to 150 eggs on day 5 of the new feed (94% drop in production). The feed was changed on day 6 and improvement in clinical signs and normal levels of egg production resumed about 12 days later. Mortality was not changed in the flock; however, necropsies of hens that died after 4 days of the new feed revealed atrophy of the ovaries and abnormally small oviducts. The authors hypothesized that the hens were exposed to a small quantity of highly contaminated trichothecene mycotoxin feed causing direct effects on the female reproductive tract and drop in egg production. The feed sample analyzed for mycotoxins contained T-2 toxin and HT-2 toxin at 3.5 and 0.7 mg/kg, respectively, which the authors thought might be unrepresentatively low. Brake *et al.* (1999) reported low levels of purified DAS ( $\leq 5$  mg DAS/kg diet) fed to broiler hens from 67 to 69 weeks of age increased fertility, with little effect on hatchability of fertile eggs. In contrast, DAS fed at 10 or 20 mg/kg diet to broiler males from 25 to 27 weeks of age decreased the hatchability of fertile eggs, which the authors attributed to direct toxic effects on the testes (and obviously on sperm). Reproductive toxicity associated with trichothecenes generally occurs when exposures reach maternally toxic concentrations, but natural trichothecene-contaminated diets can pose a serious risk to reproductive performance of livestock (Francis, 1989).

## GENOTOXICITY AND TERATOGENESIS

Trichothecenes are not mutagenic in bacterial assays, but *in vitro* exposure of Chinese hamster cells to DON at concentrations less than 1 mg/ml caused chromatid breaks (Hsia *et al.*, 1988). A 2-year chronic study in B6C3F1 mice fed diets containing 0, 1, 5, or 10 mg DON/kg diet revealed no consistent toxic effects, though mice fed higher concentrations of DON gained less weight than controls (Iverson *et al.*, 1995). DON did not consistently alter clinical chemistry or hematology parameters. The pathology revealed a reduction in development of pre-neoplastic and neoplastic lesions in the liver that may have resulted from reduced feed intake and reduction and body weights.

When considering the etiology of congenital malformation, the role of maternal toxicity must be evaluated.

Maternally toxic doses of trichothecenes can be embryo-toxic, with fetal death common in both birds and mammals, generally few frank congenital defects are observed in surviving fetuses, though anomalies in the nervous and skeletal systems have been noted (Francis, 1989). Khera *et al.* (1982) studied the embryotoxicity of DON in pregnant Swiss-Webster mice dosed orally with purified DON at concentrations of 0–15 mg DON/kg body weight for 4 days on gestational days 8 through 11. Mice dosed with 5–15 mg DON/kg body weight apparently resorbed the embryos, but no adverse effects were noticed in the dams given 2.5 mg DON/kg body weight. A number of skeletal malformations were observed in offspring of mice dosed at 1, 2.5 and 5 mg DON/kg body weight, but no adverse effects were reported in offspring of mice dosed at 0.5 mg DON/kg body weight.

DON fed to rabbits on days 0 through 30 of gestation at increasing levels of 0.3–2.0 mg/kg body weight/day caused 100% fetal resorption at 1.8 and 2.0 mg/kg/day and reduced body weight in rabbit does (Khera *et al.*, 1986). Dosages of 0.3 and 0.6 mg DON/kg body weight/day did not produce adverse effects in rabbit fetuses at term and were not maternotoxic. The authors concluded that DON did not produce a teratogenic response in rabbits. A two-generation study of female reproduction and teratology in CD-1 mice fed 0, 1.5 and 3 mg T-2 toxin/kg in a semi-synthetic diet did not reveal any significant differences in major or minor defects among treatment groups (Rousseaux *et al.*, 1986). No long-term reproductive or teratological effects were noted.

Minor malformations described as delayed ossification and un-withdrawn yolk sac were reported in chick embryos from hens fed rations containing DON at 2.5 and 3.1 mg DON/kg diet (Bergsjø *et al.*, 1993a).

## Clinical pathology

Trichothecenes, in particular T-2 toxin, have been associated with hemorrhagic syndromes; however, the effects are not consistently seen in experimental studies across species (Raisbeck *et al.*, 1991). Prolonged activated partial thromboplastin time and decreased hematocrit, leukocyte count and serum alkaline phosphatase activity were observed in rabbits injected with 0.5 mg T-2 toxin/kg body weight (Gentry and Cooper, 1981). In contrast, rabbits given 2 mg T-2 toxin/kg body weight orally displayed oral lesions, diarrhea and anorexia but no significant changes in hematological and biochemical parameters. Young mice and rats fed purified T-2 toxin at concentrations of 10–20 mg/kg for 2–4 weeks were relatively resistant to hematopoietic suppression, but developed atrophy of the thymus and thymus-dependent lymphoid tissue and lymphopenia (Hayes and Schiefer, 1982). Lambs dosed orally with 0.6 mg T-2 toxin/kg



body weight/day displayed significantly prolonged prothrombin times on day 7 (Friend *et al.*, 1983). Although lambs fed DAS (5 mg/kg diet) for 34 days showed significant decreases in several serum clinical chemistry values (cholinesterase and urea nitrogen), no changes were noted in prothrombin times, white blood count, or other hematological parameters (Harvey *et al.*, 1995). Growing pigs fed purified DAS at 0, 2, 4, 8 and 9 mg/kg diet for up to 9 weeks exhibited decreased feed consumption and weight gain (Weaver *et al.*, 1981). No changes in hemoglobin concentration, total red or white blood cell counts, and terminal bone marrow smears or in serum activities of aspartate or alanine aminotransaminases or lactate dehydrogenase were reported in the pigs. DAS given intravenously to pigs, cattle and dogs produced moderate-to-severe necrosis of bone marrow hematopoietic elements 8 h post-administration (Coppock *et al.*, 1989). The authors ranked species sensitivity to DAS destruction of bone marrow hematopoietic elements; most sensitive species were pigs and dogs and least sensitive species were cattle.

The clinical signs observed in laboratory animals treated with T-2 toxin and DAS are those of a radiomimetic poison with damage to the lymph nodes, thymus, spleen and bone marrow. A single dose of a potent trichothecene releases stored leukocytes into the blood stream but repeated administrations damage the hematopoietic system, depleting the leukocyte population. Lutsky *et al.* (1978) reported a marked depletion of leukocytes, similar to ATA, in cats dosed orally at 0.06–0.10 mg T-2 toxin/kg body weight for 24 days. T-2 toxin given intravenously to calves at 0.25 mg/kg body weight caused a marked decrease in leukocyte and neutrophil counts, with a small increase in serum aspartate aminotransferase and lactate dehydrogenase activities (Gentry *et al.*, 1984). In a study of lambs dosed orally at 0, 0.3 and 0.6 mg T-2 toxin/kg body weight for 21 days, examination of the myeloid:erythroid ratio of sternal bone marrow of the lambs revealed on day 12 that the ratio was significantly increased in 0.6 mg T-2 toxin/kg body weight lambs (Friend *et al.*, 1983). The bone marrows of these lambs appeared hypocellular with degenerating cells and pyknotic nuclei, and cells of the myeloid series predominated with few metarubricytes and more immature erythroid cells. None of the lambs were anemic and no hemorrhage was observed; nor were any significant differences found in hematological parameters. At the end of the 21-day study, the lambs were killed and necropsied. The highest dosed lambs had little body fat and pale bone marrow. In the 0.6 mg T-2 toxin/kg body weight lambs, the splenic white pulp was less cellular than low T-2 dosed and control lambs. The cortices of the mesenteric lymph nodes of T-2 treated lambs were thin, as compared with control lambs, and the lymphocyte population of the medullary and

paracortical areas and germinal centers of the mesenteric lymph nodes were markedly depleted.

Starter pigs fed T-2 toxin at 0.5, 1, 2 and 3 mg/kg diet (average daily intakes 0.38, 0.81, 1.24 and 1.42 mg T-2 toxin, respectively) for 3 weeks showed significant decreases in leukocyte counts, and pigs fed 2 and 3 mg T-2 toxin/kg had significant decreases in red blood cell counts, mean corpuscular volume and hemoglobin concentration on day 21 of the study (Rafai *et al.*, 1995b). The authors suggested that T-2 toxin affects hematopoiesis in young pigs at low concentrations (0.5 mg T-2 toxin/kg diet). In contrast, no effects on leukocyte counts or lymphoid tissue morphology were found in pigs fed 1, 2, 4, or 8 mg T-2 toxin/kg diet for 8 weeks (Weaver *et al.*, 1978a), nor were changes in hematocrit, hemoglobin concentration, or erythrocyte counts found in pigs fed 0.4, 0.8, 1.6, or 3.2 mg T-2 toxin/kg feed for 5 weeks (Friend *et al.*, 1992). However, microcytic, hypochromic anemia was reported in pigs fed higher concentrations of T-2 toxin at 10 mg/kg diet for 21–28 days (Harvey *et al.*, 1990).

Patterson *et al.* (1979) could not reproduce a feed-associated hemorrhagic disease syndrome in weaned calves or piglets by dosing orally with either purified T-2 or DAS (0.1 mg toxin/kg body weight for 36 days to pigs and 0.2 mg toxin/kg body weight for 11 days to calves) or with whole cultures of *F. tricinatum* containing T-2 toxin. While none of the pigs displayed clinical signs of toxicity, the calves dosed with 0.2 mg T-2 toxin/kg body weight did become weak, anorectic and died with evidence of a prolonged partial thromboplastin time.

Oral lesions were observed in most birds fed T-2 toxin diets, but no significant changes were observed in hemoglobin, hematocrit and erythrocyte counts in the Leghorn laying hens fed T-2 toxin (0, 0.5, 1, 2, 4 and 8 mg/kg diet) (Chi *et al.*, 1977). Serum activities of alkaline phosphatase and lactate dehydrogenase and uric acid concentrations were elevated in hens fed 8 mg T-2 toxin/kg diet, as compared with controls. In a high dose T-2 toxin study, plasma protein concentrations decreased by 15% after the first week and by 20% after the third week in hens fed T-2 toxin (20 mg/kg diet) for 21 days, as compared with control birds (Wyatt *et al.*, 1975). After receiving T-2 toxin for 3 weeks, treated hens had about 30% lower leukocyte counts than the control hens. No differences were observed in prothrombin times, erythrocyte counts, hematocrit, hemoglobin concentrations, or spleen and heart weight.

No consistent changes in serum enzyme activities have been associated with trichothecenes in animals. Low dietary concentrations of DON (<0.84 mg/kg diet) fed to weanling pigs for 4 weeks caused no significant changes in plasma enzyme activities, glucose, urea, bilirubin and hematological parameters during the study (Accensi *et al.*, 2006). Controlled studies of T-2 toxin fed to swine resulted in altered serum concentrations of



glucose and other parameters associated with decreased feed intake and changes in metabolism. Pigs fed 1, 2 and 3 mg T-2 toxin/kg feed for 3 weeks had significantly lower plasma glucose concentrations, as compared with control pigs and pigs dosed at 0.5 mg T-2 toxin/kg diet (Rafai *et al.*, 1995a). The decrease in plasma glucose was attributed to glucose malabsorption or to enhanced glucose degradation. Schiefer and Beasley (1989) suggested that in acute T-2 toxicosis in pigs, the ability of glucocorticoids to induce enzymes associated with hepatic gluconeogenesis and convert amino acids into glucose is reduced. Following an acute, intravenous toxic dose of DAS in pigs, Coppock *et al.* (1985b) reported the pigs developed hypoglycemia and depletion of liver glycogen stores. The authors attributed changes in blood glucose concentrations to cellular damage of endocrine and exocrine pancreas and adrenal gland by DAS.

## TOXICITY

Trichothecenes are toxic to all animal species that have been tested. The Type D trichothecenes, macrocyclics such as the verrucarins and roridin E, are the most acutely toxic trichothecenes, followed by the Type A compounds, DAS and T-2 toxin, Type B, nivalenol and the lowest acute toxicity is associated with the Type C trichothecene, crotoxin (Ueno, 1983). Neonatal animals are more susceptible to trichothecene adverse effects. T-2 toxin, the first trichothecene recognized as a naturally occurring mycotoxin, has been studied extensively because of its relative ease of production and its potential as a chemical warfare agent, but T-2 toxicosis is rare in North America. Oral LD<sub>50</sub> concentrations for T-2 toxin in laboratory animals did not demonstrate marked species differences in sensitivity, but agricultural species do vary in their sensitivity to the different trichothecene toxins. For example, based on toxicity, the species susceptibility to DON is ranked as pig (most sensitive), followed by rodent > dog > cat > poultry > ruminants (least sensitive) (Prelusky *et al.*, 1994).

Diarrhea is fairly common following trichothecene ingestion and altered intestinal absorption of compounds and impaired permeability is caused by morphological and functional damage to intestinal mucosa (Ueno, 1983). Epithelial irritation, particularly associated with the metabolites of *F. sporotrichioides*, is considered the underlying cause of oral and cutaneous inflammatory lesions noted in ATA, stachybotryotoxicosis and fusario-toxicosis of chickens. Dermal toxicity is common to many trichothecenes and the basis for screening toxicity of trichothecenes using dermal bioassays. The chemical structure of trichothecenes determines their dermal toxicity

and their cytotoxicity in cultured cell lines. Macrocyclic trichothecenes and T-2 toxin, HT-2 toxin and DAS (Type A trichothecenes) caused marked epithelial irritation, while nivalenol and DON (Type B trichothecenes) were weak dermal irritants in a guinea pig dermal bioassay (Ueno, 1984). Topical applications of purified T-2 toxin to various animal species caused similar morphological changes, including hyperemia, swelling, exudation and necrosis with subsequent formation of scabs that fall off (Pang *et al.*, 1989). Histological changes included vascular dilation, edema, congestion and infiltration of neutrophils, mononuclear and mast cells in the dermis with varying degrees of epidermal necrosis. The mechanism by which trichothecenes induced cutaneous damage is unknown. Ueno (1984) suggested that increased vascular permeability has a role in inducing cutaneous injury from trichothecene administration. Release of chemical mediators of inflammation could initiate changes leading to microvascular injury in dermal tissue.

In addition to the direct clinical effects on animals, Goyarts *et al.* (2005) noted in natural DON-contaminated wheat that DON significantly increased metabolizable energy, nitrogen retention digestibility of organic matter, crude protein, crude fat and crude fiber of the cereal grain. However, positive effects of a DON-contaminated diet did not compensate for adverse effects on feed intake or performance in weight gain in swine. Matthaus *et al.* (2004) reported marked changes in nutrient characteristics in *Fusarium*-contaminated wheat, with increased activities of non-starch polysaccharide hydrolyzing enzymes. This may represent a type of "pre-digestion effect" (Dänicke *et al.*, 2004b). Contrary to the positive findings of DON on feed digestibility, DiCostanzo *et al.* (1994) reviewed data reporting that moldy corn had decreased nutritional value, particularly lowered crude fat, along with energy and crude protein values.

## Swine

Clinical effects associated with DON ingestion in pigs include feed refusal, weight loss, poor performance, gastrointestinal irritation (such as diarrhea, colic and rectal prolapse), squamous hyperplasia of the gastric lining and possible immunomodulation. The DON dose affecting feed intake and rate of gain in swine studies varied with the age of the pig, source of DON (purified versus natural contamination) and exposure conditions (*ad libitum* versus restrictive feeding and duration of exposure). In a chronic study of the effects of DON on grower (starting weight of about 26 kg) and finisher swine, Goyarts *et al.* (2005) fed DON-contaminated wheat at about 6.5 mg DON/kg diet for 11 weeks in both *ad libitum* and restricted feeding regimens. Swine fed the DON-contaminated diet *ad libitum* showed

significant reductions in feed intake (15% less) and weight gain (13% less), but the feed-to-gain ratio was not affected. The lower growth performance in swine fed the DON-contaminated diet was attributed to lower voluntary feed intake because no differences were detected in weight gain for control and DON fed swine on the restricted feeding regimen. Similarly, Rotter *et al.* (1994) attributed depressed growth of growing pigs to reduced voluntary feed intake of DON-contaminated diets. Chavez and Rheaume (1986) observed that pigs fed DON-contaminated diets generally consume small amounts of feed continuously throughout the day. Pigs not only consumed less DON-contaminated feed when fed *ad libitum*, but also required more time to consume an equivalent amount of feed compared with a control group (Goyarts *et al.*, 2005).

Pollmann *et al.* (1985) fed DON-contaminated wheat at increasing concentrations of 0–2.8 mg DON/kg diet to starter pigs (7.7–8.3 kg) and concentrations of 0–4.2 mg DON/kg diet to growing/finishing pigs (60.5 kg). In the starter pigs, feed intake declined when the DON concentration was greater than 1 mg DON/kg diet, which was more noticeable during the first week of exposure. No clinical signs, including vomiting, were observed nor were lesions observed in examined organs. Vomiting was the only adverse clinical sign observed in growers fed a diet of DON at 2.8 mg/kg for 42 days. Feed refusal was observed in growers fed DON at 2.2, 2.8 and 4.2 mg/kg diet. Pigs (25 kg) fed naturally contaminated oats at 2 and 4 mg DON/kg feed experienced a dose-related depression of weight gain in the first 8 weeks of the trial (Bergsjö *et al.*, 1992). The authors determined a no-effect level based on feed intake, weight gain and feed efficiency in growing pigs (25 kg initial weight) of 0.5 and 1.0 mg DON/kg feed. In contrast, Friend *et al.* (1982) fed pigs a diet of 0.7 mg DON/kg feed and saw an immediate reduction in feed consumption, which gradually recovered to nearly normal feed intake. Significant reductions in feed intake and weight gain occurred in pigs fed corn silage naturally contaminated with 1.3 mg DON/kg feed (Young *et al.*, 1983). However, Chavez (1984) reported no reduction in weight gain or feed consumption in piglets (initial weight 7 kg) fed naturally contaminated wheat at DON concentration <2.5 mg DON/kg diet. Williams *et al.* (1988) observed that naturally DON-contaminated wheat fed to growing pigs inhibited voluntary feed intake linearly with dietary concentrations of DON up to 16 mg DON/kg feed. They also noted vomiting and signs of abdominal distress at dose rates from 0.055 to 0.097 mg DON/kg body weight. These values are close to the minimum oral emetic dose of young swine (9–10 kg) at 0.1 mg DON/kg body weight (Forsyth *et al.*, 1977).

Using naturally contaminated oats at 0, 0.7, 1.7 and 3.5 mg DON/kg feed, Bergsjö *et al.* (1993b) examined

clinical effects in growing pigs (~21 kg) fed *ad libitum* for 95 days. The daily weight gain of pigs fed 3.5 mg DON/kg feed was 82% of the control pigs over the whole experiment. Significant decreases in body weight gain, slaughter weight and feed utilization were observed in pigs fed a diet of 3.5 mg DON/kg. The authors concluded that 1.7 mg DON/kg feed, in an otherwise adequate diet which contained only minor traces of other mycotoxins, depressed daily feed intake. Dänicke *et al.* (2004b) reported no significant effects on the performance of finishing pigs when fed DON at concentrations of 0.2, 0.7, 1.2, 2.5 and 3.7 mg/kg diet.

Pigs fed higher DON-contaminated diets (>3.6 mg/kg diet) have significant weight reductions (Friend *et al.*, 1986) and reduced feed consumption (Forsyth *et al.*, 1977), as compared with controls. Significant reductions in the feed-to-gain ratio in swine were only observed at higher DON and zearalenone concentrations, 9.1 and 11.0 mg DON/kg feed and 1.2 and 2.0 mg zearalenone/kg feed, and not at lower DON concentrations (<0.5, 2.6, 5.0 and 8.3 mg DON/kg feed) when naturally contaminated wheat was fed to growing pigs for 14 weeks (Williams *et al.*, 1988). In contrast, Swamy *et al.* (2002) reported a significant decrease of about 30% in feed intake and growth performance, but not in the feed-to-gain ratio, in growing pigs fed naturally contaminated DON at 5.6 mg/kg *ad libitum* for 21 days. In another study with swine, Swamy *et al.* (2003) reported an increase in feed efficiency in pigs fed DON and suggested that pigs adjusted to the reduction in feed intake by improving feed utilization.

Based on reviews of the literature, Dänicke *et al.* (2001) suggested dietary DON concentrations in livestock feeds that ensured no adverse effects on animal health or performance if all production conditions are optimal (Table 94.2). These suggested concentrations of DON in livestock feed are similar to the U.S. Food and Drug Administration (FDA) advisory guidelines for DON in livestock feed. Eriksen and Pettersson (2004) proposed lower guidelines for DON in livestock feed based on risk assessment of data from toxic effects (Table 94.2).

Sex-related differences have been reported in pigs fed DON-contaminated feed, but the findings are equivocal. Côté *et al.* (1985) reported lower weight gains in castrated male pigs, as compared to female pigs, in the first 4 weeks of feeding DON-contaminated feeds at 3.1 and 5.8 mg DON/kg. Conversely, Goyarts *et al.* (2005) reported that *ad libitum* fed castrated male pigs had significantly higher feed intake and weight gains, as compared with female pigs, during the 11-week exposure to DON-contaminated feed (about 6 mg DON/kg diet) in grower swine; whereas Bergsjö *et al.* (1992) and Williams *et al.* (1988) reported no interactions between sex and weight gains at any dietary DON concentrations.

Rotter *et al.* (1994) concluded that pigs show a physiological adaptation response to DON-contaminated

TABLE 94.2 Recommended values for concentrations of DON in the final ration

Animal species/type	DON <sup>a</sup>	DON <sup>b</sup>	DON <sup>c</sup>
Pigs	1	1	0.3
Pre-ruminant calf		2	
Dairy cow/pregnant cattle	5	2 <sup>d</sup>	–
Beef cattle	5	5	–
Poultry (laying hens, broilers)	5	5	2.5

<sup>a</sup>Values for critical concentrations of DON in diets of pigs, ruminants, gallinaceous poultry (mg/kg, 88% dry matter) (Dänicke *et al.*, 2001).

<sup>b</sup>Values are in mg/kg for the final ration (FDA advisory guidelines).

<sup>c</sup>Risk assessment guidelines (Eriksen and Pettersson, 2004).

<sup>d</sup>Under the “intended use” dairy cattle would be in the “all other animal species.”

diets ranging from 0.95 to 2.85 mg DON/kg. Even at the lowest dose, 0.95 mg DON/kg diet (0.11 mg DON/kg body weight/day), pigs showed a reduction in feed consumption over the first 2 days of the experiment but were able to adapt by the end of 28 days. Overall gains of pigs on DON-contaminated diets were no different than pair-fed controls. This result is consistent with those of Friend *et al.* (1986) and Bergsjö *et al.* (1992) who reported that swine could adapt to dietary DON contamination. Morphological changes in pigs fed the highest DON-contaminated feed were a reduction in thyroid size and a higher degree of folding and thickening of the esophageal region of the stomach (Rotter *et al.*, 1994). In a similar study, Øvernes *et al.* (1997) fed growing pigs (25 kg) naturally DON-contaminated oats at 0.6, 1.8 and 4.7 mg DON/kg feed, both restricted and *ad libitum* for about 100 days. No changes in feed intakes were detected in pigs fed DON-contaminated rations *ad libitum*, but feed intakes were significantly reduced in pigs fed restricted levels of 1.8 and 4.7 mg DON/kg feed. Post-mortem examination of the pigs revealed no differences in the stomach mucosal appearance between the different DON-treatment groups. The authors hypothesized that when DON-contaminated feed is freely available, pigs would reject less feed than when offered large quantities for a limited period. Results of this study in growing pigs indicated that using naturally contaminated oats at levels up to 4.7 mg DON/kg feed might cause slight immunosuppression, but did not represent a serious health problem.

In a study at low T-2 toxin concentrations, Rafai *et al.* (1995a) fed 7-week-old pigs (~9 kg) a diet with 0.5, 1, 2, 3, 4, 5, 10 and 15 mg/kg of purified T-2 toxin for 3 weeks, with average daily intakes by experimental pigs of T-2 toxin between 0.029 and 0.23 mg T-2 toxin/kg body weight/day. No vomiting or diarrhea was observed in any group, but pigs fed 10 and 15 mg T-2 toxin/kg diet became somnolent and huddled as if chilled. Feed consumption was decreased (not significantly) and serum aspartate aminotransferase activity was significantly increased in pigs by the lowest T-2 toxin concentration.

Pigs fed 3.0 mg T-2 toxin/kg feed had significant decreases in weight gain, but not feed conversion, for 3 weeks. After 9–14 days on 4, 5, 10, or 15 mg T-2 toxin/kg diet, pigs began to show signs of dermatitis and crusting of the skin and the snout, with inflammation becoming more severe with time. Histological examination of affected skin revealed hyperkeratosis, parakeratosis, acanthosis and superficial to deep erosions into the sub-epithelial layer in all groups exposed to T-2 toxin. Pigs at the higher T-2 toxin dosages had involvement of the skin and mucous membranes of the prepuce, tongue and renal pelvis (urothelium). The authors of this study concluded that the no-effect level of T-2 toxin for performance was probably less than 0.5 mg T-2 toxin/kg diet. In contrast, growing crossbred pigs (18 kg) fed 8.0 mg T-2 toxin/kg diet for 30 days showed reduced feed consumption and weight gain but no changes in clinical appearance, as compared with controls (Harvey *et al.*, 1994).

Toxicoses from macrocyclic trichothecenes have occurred in pigs housed on straw litter infected with *Stachybotrys* (Dankó, 1975). The clinical signs included irritation, necrosis and hemorrhages, and are similar to those described for cattle with stachybotryotoxicosis (see Ruminants).

## Horses

Few experimental trichothecene studies using the equine species have been published. Five adult horses (~444 kg) were fed DON-contaminated barley (average range of 36–44 mg DON/kg grain) and consumed approximately 1.27 kg barley/horse/day (44–55 mg DON/horse/day or about 0.099–0.124 mg DON/kg body weight/day) for 40 days (Johnson *et al.*, 1997). The horses had access to free-choice pasture grass. No signs of feed refusal were observed in the horses, and though not weighed, all animals appeared to gain weight and improve body condition according to the authors. No remarkable changes were detected in any hematological or serum biochemical parameters. Both serum IgG and IgA decreased in a linear manner through the trial. The authors attributed decreases in serum total protein, globulin and albumin to changes in the hydration status of the animals and voluntary water consumption during the study. The authors also suggested that gastric microflora detoxified DON prior to absorption. Raymond *et al.* (2003) fed mature, non-exercising mares a blend of naturally contaminated wheat and corn (15.0 mg DON and 0.8 mg 15-ADON/kg diet), with and without 0.2% glucomannan polymer (an adsorbent), for 21 days. Control mares consumed approximately 0.004 mg DON/kg body weight/day, the mycotoxin group 0.029 mg DON/kg body weight/day, and the mycotoxin plus adsorbent group 0.051–0.058 mg DON/kg body weight/day.



Feed intake was significantly reduced in the mycotoxin-fed groups, as compared with the control group. The authors reported that  $\gamma$ -glutamyltransferase activities were increased in mares fed the mycotoxin diet on days 7 and 14, but not on day 21 of the trial. Unfortunately, no serum chemistry analyses were undertaken prior to the experiment on day 0 to establish a common baseline for the horses. Additional serum chemistry parameters and hematology were not affected by the diets. In a subsequent study of exercised horses, mares were fed one of three treatments, a control diet, a mycotoxin diet (11.2mg DON/kg, 0.7mg 15-acetyldeoxynivalenol/kg diet) and a mycotoxin diet (14.15mg DON/kg feed, 0.7mg 15-acetyldeoxynivalenol/kg diet) with 0.2% glucomannan polymer for 21 days (Raymond *et al.*, 2005). Feed intake and weight gains were depressed in horses fed the mycotoxin-contaminated diets, as compared with control mares. No effect of diet was seen on hematology or serum chemistries, which included  $\gamma$ -glutamyltransferase activities, nor were any differences noted in athletic ability measured by time-to-fatigue treadmill step tests. Feeding 2% glucomannan polymer as an adsorbent did not prevent a depression in feed consumption in this study.

Lethal trichothecene mycotoxicoses from *Stachybotrys* have been recognized in field cases involving horses in the former USSR, particularly the Ukraine since the late 1930s (reviewed by Forgacs, 1972; Dankó, 1975; Hintikka, 1978). *Stachybotryotoxicosis* typically occurred during indoor feeding of horses with *S. alternans*-contaminated straw or hay. The horse is very sensitive to *Stachybotrys* toxins, with 1mg of pure *Stachybotrys* toxin considered a lethal dose. Soviet authors described two forms of the disease, "typical" or prolonged disease and "atypical" or acute disease, based on the dose and duration of toxin ingested. The typical form, which is further subdivided into three stages, was more common, and occurred after continuous consumption of sub-lethal quantities of moldy vegetation. The first stage occurred 2 or 3 days following consumption of moldy feed. Irritation, and later necrosis, occurred wherever the toxin(s) contacted tissue, especially the oral, nasal and ocular mucous membranes. Clinical signs of conjunctivitis, lacrimation and profuse salivation, along with spontaneous nosebleeds, hemorrhage of mucous membranes, maxillary lymph node swelling and dysphagia, also occurred. The first stage lasted 2–3 days or could persist for a month. The second stage lasted 15–50 days and was characterized primarily by clinicopathological changes; clinical signs were non-specific. An initial leukocytosis was followed by leukopenia and thrombocytopenia and, in some cases, blood failed to clot. During the third stage, thrombocytopenia and leukopenia worsened, horses became febrile (40–41.5°C), anorexic and prone to colic. Weight loss, recumbency and septicemia were

common. This stage lasted 1–6 days and usually ended in death.

The atypical form of *stachybotryotoxicosis* was peracute (1–3 days) and associated with high concentrations of *Stachybotrys* toxins in the feed. This form was characterized by nervous system irritation or depression, cardiac arrhythmias, pulmonary edema and hemorrhages of serosal and mucosal membranes and muscular tissue, muscle necrosis and oral ulcers. Post-mortem findings were characterized by hemorrhage and necrosis of tissues (Hintikka, 1978). Hemorrhages occurred on serous and mucous membranes and in the spleen, liver, lungs, brain, spinal cord, lymph nodes and most notably in muscle tissue. Clearly circumscribed necrotic foci, yellow to brown-gray in color, were seen on oral mucosa, with the entire digestive tract generally appearing hemorrhagic and necrotic.

In addition to toxicoses involving macrocyclic trichothecenes, historical accounts of equine deaths were reported in horses provided moldy feed in northern Japan. Horses developed clinical signs and died after consuming moldy bean hulls, which were commonly used as fodder and bedding especially in the winter and spring (Ishii *et al.*, 1971). The clinical signs of affected horses were central nervous system disturbances including convulsions and cyclic movements, depressed respiration, icterus and bradycardia. Several trichothecenes, including T-2 toxin, DAS and neosolaniol, were associated with the clinical syndrome, but cause-effect was not established.

## Dogs and cats

Trichothecenes are fairly heat stable compounds and following incorporation into pet food diets, DON remains stable during extrusion and drying (Hughes *et al.*, 1999). The authors reported that DON concentrations (from a naturally contaminated wheat source) greater than  $4.5 \pm 1.7$  or  $7.7 \pm 1.1$ mg DON/kg diet reduced feed intake in dogs and cats, respectively. When DON concentrations reached 8–10mg/kg feed, clinical signs of vomiting and reduced feed intake and body weight occurred in dogs and cats. Dogs that had been previously exposed to DON-contaminated feed preferentially selected uncontaminated dog food; dogs with no previous experience with DON-contaminated dog food consumed equal quantities of DON-contaminated and uncontaminated food.

## Ruminants

During wet growing seasons, DON contamination of cereal grains can be sufficiently high to make grains unsuitable for human food or swine feed. Anderson *et al.* (1996) evaluated the use of naturally scab-infected barley



with elevated DON concentrations (about 35mg/kg) in beef growing/finishing and pregnant heifer rations. The authors fed increasing levels of DON to crossbred steers for 84 days, at 0.9, 3.7, 6.4 and 9.2mg DON/kg diet, during the growing period and for 100 days during the finishing period at 1.1, 5.0, 8.8 and 12.6mg DON/kg diet. No significant differences were found in beef performance including feed intake, feed efficiency, rate of gain, or carcass quality. The authors concluded that DON-contaminated feed could be provided up to 9mg/kg diet during bovine growth and up to 12mg/kg diet during finishing without adverse effects. In a separate study, the authors fed pregnant yearling heifers DON-contaminated barley, 36.8mg/kg at 3.6 or 5.4kg/head/day (about 10.2 or 13mg DON/kg diet, respectively) from mid-gestation through the first 45 days of lactation. No significant changes were noted in feed intake, heifer weight gain, or calf body weights. Calf weight gains were higher for calves nursing heifers fed the DON-contaminated barley during calving and lactation. In a 3-year follow-up of the experimental heifers retained in the herd, all 14 control heifers had been culled for non-pregnancy after 3 years, whereas only eight vomitoxin-fed heifers had been culled for non-pregnancy suggesting feeding DON had no impact on subsequent reproduction (Anderson, 2006). The authors concluded that barley unsuitable for human food could be utilized in beef rations and fed safely at 10–12mg DON/kg diet to pregnant cows.

Trenholm *et al.* (1985) reported an initial slight decrease in feed intake of dry cows fed a wheat–oats-contaminated diet with 6.4mg DON/kg concentrate. The cows returned to normal feed consumption by the end of the sixth week of exposure, with no differences reported in body weight. The only trichothecene mycotoxin apparently tested in the contaminated wheat was DON, so the presence of additional mycotoxins could not be discounted. Charmley *et al.* (1993) fed primiparous Holstein cows DON-contaminated wheat and corn at increasing concentrations of 0.07, 4.95 and 12.09mg DON/kg of dry matter concentrate (daily DON intake of 0.59, 42 and 104mg, respectively) for 10 weeks in a lactation study. The total mixed ration was formulated to support 25kg milk/day and a milk fat level of 3.8%. No changes were observed in total milk output or intake of concentrate or forage. Cows fed 4.9mg DON/kg concentrate had the lowest milk fat content, which the authors did not attribute to DON intake. Similar results were reported by Ingalls (1996) who fed high-producing dairy cows DON-contaminated barley diets up to 14.6mg DON/kg concentrate dry matter (about 8.5ppm in diet, 0.31mg DON/kg body weight) for 3 weeks and found no significant changes in milk production or feed intake between controls and treated cows.

Oral T-2 toxin dosing studies in calves resulted in more severe clinical signs. Pier *et al.* (1976) dosed young Jersey

calves, 35–78kg, with oral gelatin capsules for 30 days at 0.08, 0.16, 0.32 and 0.64mg T-2 toxin/kg body weight (one calf per dose level). The authors estimated that the lower T-2 doses were realistic, as a 50kg calf would have to eat 2kg of corn containing 2mg T-2 toxin/kg to mimic the lowest dose of 0.08mg T-2 toxin/kg feed. All T-2 toxin dosages caused mild enteritis and loose feces in calves. At the higher dosage levels of 0.32 and 0.64mg/kg, bloody feces were apparent, and the calves became anorexic, dehydrated and lost body weight. The calves had elevated serum aspartate aminotransferase activities and prolonged prothrombin times. The high dose calf died on day 20 of the trial. Post-mortem lesions in the highest T-2 toxin dosed calves were ruminal ulcers and abomasitis, while the lower dosed calves had mild enteritis. Abomasal ulcers were described in the calf dosed at 0.16mg T-2 toxin/kg body weight.

Oswailer *et al.* (1981) dosed beef calves (85–200kg) orally with 0.1, 0.3 and 0.6mg purified T-2 toxin/kg body weight/day for 6 weeks, and found that 0.3mg/kg (mimic ~10mg T-2/kg diet) decreased feed consumption with no outwards signs of clinical disease. A clinically effective dose of 0.6mg/kg (mimic ~20mg T-2/kg diet) produced marked differences in body weight gain, as compared with control animals. All calves in the high dose group (0.6mg T-2 toxin/kg body weight) refused to eat the concentrate portion of their diet starting on the second day of the trial and consumed a limited portion of diet provided throughout the entire 6 weeks of T-2 toxin exposure. The high T-2 toxin dosed calves had intermittent diarrhea, soft tan to dark, semi-fluid feces, and became thin with rough hair coats. Calves dosed with 0.3mg/kg generally showed a lack of interest in eating the total amount of feed provided. No coagulopathies were observed in the T-2 toxin-treated calves. The authors questioned whether cattle would consume T-2-toxin-contaminated feed in the range of 10–20mg/kg for a sufficient time to produce clinical signs.

Weaver *et al.* (1977) intubated daily a pregnant Holstein cow (third trimester of gestation) with 182mg of purified T-2 toxin for 15 days. On the fourth day of dosing, the cow delivered a bull calf that was also intubated with T-2 toxin at 0.6mg/kg body weight for 7 consecutive days, and then on alternate days for a total of 16 days of treatment. The calf became severely depressed, with hind-quarter ataxia, knuckling of rear feet and anorexia. Complete blood counts, serum total protein and several liver enzymes were within the normal range for both cow and calf throughout the treatment. Gross and microscopic lesions were seen only in the cow and included congestion of the small intestine mucosa and a ruminal ulcer. Histological changes were congestion of the lamina propria blood vessels of the omasum, rumen, jejunum and ileum, and edema of the submucosa, muscular layers and serosa of the cecum and colon. The bone marrows of both animals were normal.

In a field case, anorexia, periodic elevated temperatures, abortions in mid-gestation and a 20% death loss were associated with feeding moldy, high moisture corn to lactating Holstein dairy cows during a 5-month period in late winter (Hsu *et al.*, 1972). Post-mortem lesions of extensive hemorrhages on serosal surface of internal viscera were reported. Among the fungi cultured from the corn were *F. tricinctum*, *F. roseum* and *F. moniliforme* and various *Penicillium* spp. A sample of feed taken in late May contained 2mg T-2 toxin/kg, which the authors estimated as a low concentration because of losses during analysis. The authors assumed other toxins were also present and contributed to clinical toxicity, but were not analyzed.

Stachybotryotoxicosis in cattle occurred mainly as large outbreaks in young animals (Dankó, 1975; Hintikka, 1978). Clinical signs included lethargy, inappetance, bloody diarrhea and epistaxis. In chronic toxicosis, changes in the hematology appeared to be the initial toxic event. After a leukocytosis of short duration, leukopenia and thrombocytopenia developed. Clinical signs appeared gradually with hematological changes and were similar to those seen in the "typical" form of horses. Cows could abort during the second half of gestation with hemorrhagic lesions apparent in the fetus and fetal membranes. Mortality could be as high as 90–100% in affected cattle. Toxicosis in sheep was similar to cattle. Additionally, sheep exhibited hyperthermia and progressive anemia with marked impairment of the lymphoid system (Schneider *et al.*, 1979) and wool loss with skin hemorrhaging (Hajtós *et al.*, 1983).

Dankó (1975) commented that animals preferred to eat *Stachybotrys*-contaminated straw rather than good-quality hay because of a flavor produced by the mold in the straw, but this observation was not supported in other literature. *Stachybotrys* toxins appeared more toxic in an acidic pH (Dankó, 1975). Therefore, ruminants were considered relatively resistant because of salivation and the higher pH environment of the rumen. Feeding a high carbohydrate diet, which lowers rumen pH, could enhance the toxic effects of *Stachybotrys*. Treatment of animals affected with *Stachybotrys* mycotoxins was generally unrewarding due to severe damage to the lymphoid system. Immunosuppression of both humoral and cellular systems was an important clinical effect from toxins of *Stachybotrys*.

Ovine responses to trichothecene exposure are similar to cattle. Seven crossbred lambs (mean weight of 17.9kg) between 3 and 6 months of age consumed a DON-contaminated wheat ration (15.6mg DON/kg) for 28 days with no significant changes in behavior, clinical signs, feed consumption, hematology, or serum clinical chemistries compared to a control group (Harvey *et al.*, 1986). In an experiment using purified DAS, crossbred ewe lambs (average 38kg) were fed *ad libitum* 5.0mg DAS/kg diet for 34 days (Harvey *et al.*, 1995). The lambs

developed diarrhea during the first week of the study, with a significant decrease in feed intake and weight gains by the end of the exposure period. No dermal or oral lesions were observed in DAS treated lambs, nor were gross lesions observed at necropsy of the lambs. The authors fed lambs a diet contaminated with both aflatoxin at 2.5mg/kg feed and DAS at 5.0mg/kg feed and noted a synergistic response in the marked reductions in feed intake and weight gains.

Friend *et al.* (1983) dosed 6–8-week-old male lambs with purified T-2 toxin in gelatin capsules at 0, 0.3 and 0.6mg/kg body weight for 21 days. All lambs had bouts of diarrhea, but several lambs dosed with 0.6mg T-2 toxin/kg body weight had prolonged periods of diarrhea, appeared unthrifty and started fleece-pulling at the end of 3 weeks of treatment. At necropsy, T-2 treated lambs appeared to have smaller and less active thymic tissue, as compared to the control group, and lesions of focal rumenitis with parakeratosis and infiltration by polymorphonuclear cells.

## Poultry

Poultry are not as sensitive to the effects of trichothecenes as swine, but are probably more sensitive than ruminants. Day-old chickens fed DON-contaminated feed at 9 or 18mg DON/kg diet for 7 weeks had decreased relative and absolute liver weights, but showed no changes in feed intake (Kubena *et al.*, 1985). Chickens fed high DON concentrations in rations (>15mg DON/kg) exhibited feed refusal and weight loss (Kubena *et al.*, 1987; Kubena and Harvey, 1988). DON contamination of rations up to concentrations of 18mg/kg feed provided to laying hens did not affect hatchability of eggs (Lun *et al.*, 1986; Bergsjö *et al.*, 1993b). Bergsjö *et al.* (1993a) fed a naturally contaminated oat diet to White Leghorn laying hens at concentrations of 0.12–4.9mg DON/kg feed for 70 days. Low concentrations of 3-ADON (0–0.63mg/kg) and zearalenone (trace to 0.55mg/kg) were detected in the diets. No significant differences in feed intake, weight gain, egg production, body weight at hatching and viability of the chicks were found between treatment groups.

Purified T-2 toxin added to a commercial laying hen ration at 20mg T-2 toxin/kg diet for 3 weeks caused raised oral lesions, yellowish-white to gray in color, during the first week of T-2 toxin administration on the sublingual and palatine areas, and then on the tongue and corners of the mouth (Wyatt *et al.*, 1975). Feed consumption by hens decreased by about 25% compared with control diet intake during the first week of T-2 toxin exposure, with the treated laying hens showing a significant weight loss after 1 week of the trial. Egg production of the T-2 toxin-treated hens declined by 20% in the second and the third week of exposure; the eggshell

thickness was significantly decreased after 3 weeks of T-2 toxin exposure.

In a study of 27-week-old laying hens fed *ad libitum* diets containing purified T-2 toxin at 0 (control), 0.5, 1, 2, 4 and 8 mg/kg feed for 8 weeks, the highest dosage caused a significant decrease in feed consumption, egg production and shell thickness, as compared with hens fed the control diet (Chi *et al.*, 1977). Fertility and progeny performance were not altered by feeding T-2 toxin, but hatchability of fertile eggs was significantly decreased in hens fed 2 and 8 mg/kg, as compared with hens fed the control diet. Oral lesions, circumscribed and proliferative plaques, were noticed after the second week in hens fed 4 and 8 mg/kg and after the third week in hens fed 0.5 mg/kg. Severity and incidence of lesions were proportional to dietary T-2 toxin concentration. No significant histopathological changes were observed in the liver, heart, kidney, brain, or bone marrow, but necrotic lesions were observed in the gizzard and crop. Diaz *et al.* (1994) fed hens both T-2 toxin and DAS, each at 2 mg/kg feed in the diet. Hens exhibited oral lesions, a significant decrease in feed consumption (decline of about 12%) and egg production (decline of 15%), as compared with controls.

Generally, concentrations of T-2 and HT-2 toxin greater than 1 mg/kg are not common in grains except in wet years in the upper Midwest of the United States; often, HT-2 toxin is found at higher concentrations than T-2 toxin. Rafai *et al.* (2000) evaluated such relatively low levels (0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3 and 4 mg T-2 toxin/kg diet) of exposure for 7 weeks in day-old Pekin ducklings. Within 2 days after starting the experiment, most ducks fed T-2 toxin developed oral lesions. The dermatotoxic lesions gradually disappeared in the ducks fed less than 3 mg T-2 toxin/kg feed. Ducks fed 3 and 4 mg T-2 toxin/kg feed refused the diet in the first week and from weeks 3 to 7. The feed intakes of ducks fed 0.6–4 mg T-2 toxin/kg feed were generally less than that of the control group. No significant dose-related changes were noted in serum chemistry and hematology. Similar dose-related oral lesions were reported in day-old Muscovy ducks within 16 h of exposure to T-2 toxin and DAS at 0.25, 0.5, or 1 mg/kg feed (Shlosberg *et al.*, 1986). Decreased body weights, thymus, spleen and bursa of Fabricius weights were noted in 6-week-old mallards fed 2 mg T-2 toxin/kg feed for 9 days (Neiger *et al.*, 1994). Microscopic lesions were observed in lymphoid organs and included a moderate decrease in thymic cortical lymphocytes.

### New trichothecene concern

In North America during the mid-1990s, interest focused on *Stachybotrys chartarum* or black mold found in damp buildings as a possible cause of idiopathic

pulmonary hemorrhage in infants (Dearborn *et al.*, 1999). Epidemiological investigations of pulmonary hemorrhage in infants found an association with airborne fungi including *Stachybotrys*. *S. chartarum* produces a variety of secondary fungal metabolites, including simple trichothecenes (trichodermol, trichodermin and verrucarol), macrocyclic trichothecenes (verrucarins B and J; roridins D, E, isoE and L-2; the satratoxins F, isoF, G, isoG, H and isoH) and spirocyclic drimanes that are recognized immunosuppressants (Jarvis, 2003). *S. chartarum* appears to divide into two chemotypes, one produces the macrocyclic trichothecenes and the other produces a class of diterpenoids, the atranones that to date have not been shown to possess significant biological activity (Jarvis, 2003). The characterization of natural products from *Stachybotrys* species and any relationship to clinical disease remains a challenge.

## TREATMENT

The first action is to stop exposure to moldy feed. If using screenings or poor-quality grain, cleaning the grain by removing broken, shriveled kernels and washing the grain can lower mycotoxin contamination. Generally, clinical signs of feed refusal will disappear within 7 days after removal of the contaminated feed. Animals return to production within 14 days.

No specific therapies for trichothecene mycotoxicoses are available. Some trichothecenes undergo enterohepatic recirculation and are excreted in the feces. The use of activated charcoal, which binds toxins within the gastrointestinal tract and prevents toxin reabsorption, plus magnesium sulfate, was beneficial as part of a therapeutic protocol for treatment of acute, T-2 toxicosis in swine (Poppenga *et al.*, 1987). Metoclopramide, given intravenously immediately prior to and following T-2 toxin administration, was only partially successful as an anti-emetic (Poppenga *et al.*, 1987). A number of binders, such as clay and zeolitic products, have been suggested for use with trichothecene-contaminated feed to prevent absorption by animals. Their efficacy has not been proven and marked species variations exist. The U.S. Food and Drug Administration has not approved any ingredient for use as a trichothecene mycotoxin binder.

## CONCLUSIONS

Trichothecene mycotoxins occur worldwide; however, both total concentrations and the particular mix of toxins present vary dramatically with environmental



conditions. Proper agricultural practices such as avoiding late harvests, removing overwintered stubble from fields, and avoiding a corn/wheat rotation that favors *Fusarium* growth in residue can reduce trichothecene contamination of grains. Storage of grains at less than 13–14% moisture (less than 0.70  $a_w$ ) and hay/straw at less than 20% moisture are important in preventing trichothecenes production. Once produced, trichothecenes are stable compounds and can remain present at toxic concentrations in feed for years. Field reports of trichothecene toxicity in livestock in North America generally involve DON contamination of feed. Acute, high dose exposures are not common. More typically, chronic to sub-chronic exposure to low dose DON-contaminated feed in swine occurs and leads to nutritional impairment, poor production and possible immune susceptibility to infections.

Due to the vague nature of toxic effects attributed to low concentrations of trichothecenes, a solid link between low level exposure and a specific trichothecene(s) is difficult to establish. Mold identification and determination of fungal spores can indicate feedstuffs of deteriorated quality, but provide no information as to the presence or concentration of mycotoxins that actually cause adverse health effects. Multiple factors, such as nutrition, management and environmental conditions, impact animal health and need to be evaluated with the knowledge of the mycotoxin(s) and concentrations known to cause adverse health effects. Future research evaluating the impact(s) of low level exposure on livestock may clarify the potential impact on immunity.

Trichothecenes are rapidly excreted from animals, and residues in edible tissues, milk, or eggs are likely negligible. In chronic exposures to trichothecenes, once the contaminated feed is removed and exposure stopped, animals generally have an excellent prognosis for recovery (Prelusky *et al.*, 1994).

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# Zearalenone

Michelle S. Mostrom

## INTRODUCTION

Zearalenone is a nonsteroidal estrogenic mycotoxin produced by several species of *Fusarium* fungi. The primary producer of zearalenone is *Fusarium graminearum* (teleomorph *Gibberella zeae*). Additional *Fusarium* fungi capable of producing zearalenone include *F. culmorum*, *verticillioides* (*moniliforme*), *sporotrichioides*, *semitectum*, *equiseti* and *oxysporum*. Contamination of cereal grains by zearalenone has been reported worldwide, primarily in temperate climates. Typically, zearalenone concentrations are low in grain contaminated in the field, but increase under storage conditions with moisture greater than 30–40%. Zearalenone has major effects on reproduction that can lead to hyperestrogenism. Prepubertal swine are the most sensitive species. Typical clinical signs of hyperestrogenism are swelling of the vulva, increase in uterine size and secretions, mammary gland hyperplasia and secretion, prolonged estrus, anestrus, increased incidence of pseudopregnancy, infertility, decreased libido, and secondary complications of rectal and vaginal prolapses, stillbirths and small litters. This chapter describes toxicity of zearalenone and its major metabolites in animals.

## BACKGROUND

Estrogenism in swine was reported in the mid-1920s in the Midwest U.S. (McNutt *et al.*, 1928). A condition of swelling and eversion of the vagina in young gilts and swelling of the prepuce in males was associated with

consuming moldy corn. Prolapse of the vagina and occasionally the rectum were noted as secondary effects. With replacement of the moldy corn with clean corn, the animals recovered to normal, but if exposure to moldy corn continued, eversion of the uterus, secondary infections and death occurred. Mirocha *et al.* (1971) noted high death losses in some herds and hypothesized that mortality was related to vaginal and rectal prolapses and subsequent septicemias; however, the presence of additional mycotoxins could not be excluded in causing death. Stob *et al.* (1962) isolated an active metabolite with uterotrophic and anabolic activities from culture of *G. zeae* (*F. graminearum*).

The *Fusarium* compound found in corn with uterotrophic activity was named zearalenone, previously known as F-2, and is chemically described as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)- $\beta$ -resorcylic acid lactone (Figure 95.1). At least seven derivatives of zearalenone have been found that naturally occur in corn. Zearalenone and uterotrophic active derivatives were classified as estrogens in the sense that they produce estrus or cornification of the vagina of adult mice (Mirocha and Christensen, 1974). Zearalenone can be produced on numerous substrates, including wheat, barley, corn, corn silage, rice, sorghum and occasionally in forages. Production in soybeans is uncommon. Moisture content and the presence of oxygen are critical factors for zearalenone production. In laboratory cultures, *Fusarium* growth occurs during a 3-week period at moisture levels over 20% and temperatures between 20 and 25°C. If the fungus is stressed by cool temperatures of 8–15°C for several weeks, zearalenone can be produced. Production of zearalenone in the field is similar to that in the laboratory, with alternating moderate and low temperature

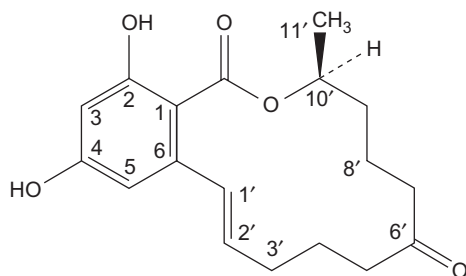


FIGURE 95.1 Chemical structure of zearalenone.

weather capable of stimulating zearalenone production. Zearalenone can be produced fairly quickly in the field during wet weather in the late summer or early fall weather following hail damage to corn. Very high concentrations of zearalenone, which can occur naturally in some field samples, generally result from improper storage at high moisture rather than production in the field. Corn stored in a crib and exposed to winter weather was particularly prone to fungal invasion and production of zearalenone. In addition to the co-occurrence of other estrogenic metabolites, such as  $\alpha$ - and  $\beta$ -zearalenol, zearalenone is commonly detected in grains with another *Fusarium* mycotoxin deoxynivalenol. Zearalenone is heat stable, but can be partially destroyed during extrusion cooking of cereals (Castells *et al.*, 2005).

## TOXICOKINETICS

Zearalenone is fairly rapidly absorbed following oral exposure (Dailey *et al.*, 1980). Following a single oral dose of 10mg zearalenone/kg body weight to 15–25kg pigs, the absorption was approximated to be 80–85% (Biehl *et al.*, 1993). Zearalenone and associated metabolites were found in the plasma of a pig in less than 30min after initiating feeding with parent compound. Following zearalenone administration, zearalenone can be localized in reproductive tissues (ovary and uterus), adipose tissue and interstitial cells of the testes (Ueno *et al.*, 1977; Kuiper-Goodman *et al.*, 1987). The reported biological half-life of total plasma zearalenone radioactivity following oral dosage in pigs is 86h (Biehl *et al.*, 1993). Species differences in zearalenone susceptibility might be related to hepatic biotransformation, with the highest amount of  $\alpha$ -zearalenol, which has increased estrogenic activity compared to  $\alpha$ -zearalanol and zearalenone produced by pig hepatic microsomes, whereas chicken microsomes produced the highest amounts of  $\beta$ -zearalenol, which has lower estrogenic activity (Malekinejad *et al.*, 2006). Pigs readily conjugated almost all absorbed zearalenone and  $\alpha$ -zearalenol through glucuronidation.

While the liver plays a major role in glucuronidation, the intestinal mucosa is also active. Zearalenone was reduced to  $\alpha$ - and  $\beta$ -zearalenol in sow intestinal mucosa homogenates (duodenum and jejunum) *in vitro* (Olsen *et al.*, 1987). Gastrointestinal flora can aid in the metabolism of zearalenone. Zearalenone can undergo rumen metabolism, with reduction to mostly  $\alpha$ -zearalenol and to a lower amount of  $\beta$ -zearalenol (Kiessling *et al.*, 1984). Whether rumen metabolism will increase or decrease zearalenone toxicity depends on absorption by the gastrointestinal tract, liver metabolism by hydroxysteroid dehydrogenase and competition at the cytosolic receptor sites in the animal species.

Zearalenone undergoes extensive enterohepatic circulation and biliary excretion in most species. The major route of excretion for most species is through the feces, although rabbits primarily excrete zearalenone in the urine. Most zearalenone administered in a dose is excreted within a 72h period. Approximately 94% of radio-labeled zearalenone, given orally to White Leghorn laying hens at 10mg/kg body weight, was eliminated through the excreta within 72h post-dosing (Dailey *et al.*, 1980). No major retention of radio-labeled activity was found in edible muscle tissue, but lipophilic metabolite(s) were reported in egg yolk (at about 2mg/kg concentration) 72h post-dosing.

Concern has focused on potential residue of zearalenone and its metabolites in milk, eggs and foods, and precocious development of sexual characteristics in young girls (Kuiper-Goodman *et al.*, 1987; JECFA, 2000). Zearalenone and  $\alpha$ - and  $\beta$ -zearalenols can be transmitted into the milk of sheep, cows and pigs administered high doses of zearalenone (Hagler *et al.*, 1980; Mirocha *et al.*, 1981). Hyperestrogenism has been reported in lambs and pigs nursing dams dosed with zearalenone (Hagler *et al.*, 1980; Palyusik *et al.*, 1980). Dairy cows fed rations with purified zearalenone at 50mg zearalenone/day and 165mg zearalenone/day for 21 days had no detectable concentrations of zearalenone or  $\alpha$ - and  $\beta$ -zearalenol in the milk or plasma (Prelusky *et al.*, 1990). One cow dosed with 544.5mg zearalenone/day for 21 days had maximum concentrations of 2.5ng zearalenone/ml and 3.0ng  $\alpha$ -zearalanol/ml in the milk. Cows dosed orally with a 1-day dose of 1.8 or 6g zearalenone had maximum milk levels on day 2 of 4.0 and 6.1ng zearalenone/ml, respectively. This research indicates that minimal transmission of zearalanone occurs into milk and only for a short period of time after exposure to high concentrations of zearalenone.

Following intubations of tritiated-zearalenone into the crops of 7-week-old broiler chickens, the greatest accumulation of radioactivity occurred in the liver 30min post-administration, which became a trace of radioactivity by 48h post-administration (Mirocha *et al.*, 1982). Only zearalenone was detected in muscle tissue at

approximately 4ppb at 48h post-administration, indicating the zearalenone residues in edible tissue are minimal.

## MECHANISM OF ACTION

Zearalenone undergoes reduction of the 6' ketone to a secondary alcohol, which leads to two diastereoisomeric zearalanols ( $\alpha$  and  $\beta$ ), which are naturally occurring fungal metabolites. The  $\alpha$ -zearalanol metabolite, which is less estrogenic than  $\alpha$ -zearalanol but three times more estrogenic than zearalenone, is an anabolic growth-promoting compound, zearanol or Ralgro<sup>®</sup>, used in both cattle and sheep commercially.

Zearalenone and metabolites can interact directly with the cytoplasmic receptor that binds to 17 $\beta$ -estradiol and translocate receptor sites to the nucleus (Katzenellenbogen *et al.*, 1979). In the nucleus, stimulation of RNA leads to protein synthesis and clinical signs of estrogenism. Following subcutaneous injection of the compounds, the zearalanols and zearalenone stimulated production of a specific uterine protein and increased uterine weights. Within the resorcylic acids,  $\alpha$ -zearalenol exhibited the greatest binding affinity for cytosolic estrogen receptors, while zearalenone and  $\beta$ -zearalenol displayed much lower binding affinities (Fitzpatrick *et al.*, 1989). The hydroxylation of zearalenone to  $\alpha$ -zearalenol apparently is an activation process, whereas the production of  $\beta$ -zearalenol would be a deactivation process. The relative binding affinity of  $\alpha$ -zearalenol was greater in the pig than in the rat or chicken. Interspecies variations in sensitivity to zearalenone in the feed could be related to different metabolites produced and the relative binding affinities of zearalenone and metabolites formed.

Zearalenone can also act on the hypothalamic-hypophyseal axis. Using 70-day-old Yorkshire gilts (20–27kg) fed 1.5–2mg zearalenone/kg feed for 45–90 days, Rainey *et al.* (1990) determined that prepubertal exposure to zearalenone affected the hypothalamic-hypophyseal axis and the luteinizing hormone (LH) surges that lasted for at least 44 days post-exposure. However, zearalenone consumption did not delay the onset of pubertal estrus or impair conception rates, ovulation rates, or number of fetuses. Slightly older prepubertal gilts (178 days of age and 94kg) fed 10mg zearalenone daily for 2 weeks had suppressed mean serum concentrations of LH, but the onset of puberty and subsequent reproduction were not adversely affected (Green *et al.*, 1990). Male rats, 70 days old, dosed orally with zearalenone at 20mg/kg body weight for 35 days, had elevated serum prolactin concentrations but showed no changes in serum LH and follicle stimulating hormone concentrations, body and testes weights,

or in spermatogonia, spermatocytes and spermatids (Milano *et al.*, 1995). At relatively high concentrations *in vitro*, approximately 400 $\mu$ M, zearalenone appeared to act directly on interstitial cells of the testes inhibiting steroidogenesis (Fenske and Fink-Gremmels, 1990).

While zearalenone primarily affects reproduction, it may have additional effects. During exposure of *in vitro* cell lines, zearalenone acted as a ligand for human pregnane X receptor (hPXR), which can activate a transcription factor regulating the expression of numerous hepatic drug-metabolizing enzymes, including expression of cytochrome P450 enzymes (Ding *et al.*, 2006). This suggests a potential for zearalenone to induce metabolism of drugs.

At natural contamination levels in feeds, zearalenone does not appear to impact the immune response. Results from *in vitro* studies of zearalenone and metabolites  $\alpha$ - and  $\beta$ -zearalenol and  $\alpha$ - and  $\beta$ -zearalanol and mitogen testing with leukoagglutinin, concanavalin A and pokeweed revealed that these mycotoxins inhibited mitogen-induced proliferation of both B and T lymphocytes (Forsell and Pestka, 1985). No treatment differences were observed in B6C3F1 mice fed a diet with 10mg zearalenone/kg diet for 8 weeks and control mice that were tested with a splenic plaque-forming response to sheep erythrocytes and a delayed hypersensitivity response to keyhole hemocyanin (Pestka *et al.*, 1987).

## TOXICITY

Zearalenone has low acute toxicity in most species. In most natural conditions, concentrations of zearalenone in feed ingredients are less than 20mg/kg (ppm) and generally less than 5mg zearalenone/kg feed (Sundlof and Strickland, 1986). Prepubertal swine are most sensitive to zearalenone, ruminants may exhibit some adverse effects, and poultry appear to be the least sensitive species. Females are more sensitive than males, and cycling female pigs may be more sensitive than pregnant sows. Pregnant swine may abort. Abortions have been associated in field cases with natural *Fusarium* mold exposure, but have not been reproduced with purified zearalenone (Mirocha and Christensen, 1974). Younger male pigs appear to be more sensitive than older males and can undergo atrophy of the testes and enlargement of mammary glands.

### Swine

Gilts fed rations with 0, 3, 6, or 9mg purified zearalenone/kg feed that started the day after they showed the first estrus were bred at subsequent heat periods



(Young and King, 1986a). A majority of gilts fed 6 or 9 mg zearalenone/kg feed became pseudopregnant based on examination of their reproductive tracts or plasma progesterone levels. Gilts fed rations with 3 mg zearalenone/kg feed had no reproductive effects. After removal of zearalenone from the diet, approximately half of the gilts fed 6 or 9 mg zearalenone/kg feed returned to estrus spontaneously. Edwards *et al.* (1987a) reported luteal maintenance and extended inter-estrous intervals in sexually mature gilts fed purified zearalenone at concentrations of 5–10 mg/kg feed from days 5 to 20 of the estrous cycle. Approximately 86% of the retained corpora lutea underwent spontaneous regression and most gilts came into estrus within the next 30 days. In a subsequent study, Edwards *et al.* (1987b) fed prepubertal gilts a diet with 0 or 10 mg zearalenone/kg feed for 30 days from 145 to 193 days of age, switched the gilts to a control diet, and then exposed the gilts to a mature boar. While treated gilts displayed vulvar swelling during the 30-day feeding period and were delayed in showing first estrus, the proportion of gilts showing estrus with exposure to the boar was similar between treatment and control gilts, and subsequent cycling was not affected when the treated feed was removed. Young *et al.* (1990) reported an increased weaning-to-estrus interval and embryonic mortality (measured as a decreased ratio of fetuses to corpora lutea) and a decreased number of fetuses per sow in second parity sows fed 10 mg zearalenone/kg diet. No reproductive effects were observed in prepubertal gilts fed a ration with 0.5 mg zearalenone/kg feed (Friend *et al.*, 1990).

Young gilts (30–35 kg) administered 5 mg of purified zearalenone *per os* daily developed swelling of the vulva on the fourth day of treatment, an approximate daily dose of 0.143–0.167 mg zearalenone/kg body weight (Mirocha and Christensen, 1974). Gilts dosed with 1 mg of purified zearalenone daily for 8 days developed pronounced vulvar swelling. Gilts exposed to higher concentrations of zearalenone may show atrophy of the ovaries along with edema and cellular proliferation of all layers in the uterus. Kuiper-Goodman *et al.* (1987) noted a no adverse effects level for zearalenone in pigs reaching puberty at 0.06 mg zearalenone/kg body weight/day.

Male swine fed a high concentration of zearalenone (30 mg/kg feed) appeared initially to have accelerated maturation of spermatogenesis, which occurred 1.5–2 months earlier than control animals (Ványi and Széky, 1980). Although germinal epithelium damage was limited to several foci initially, with continued zearalenone exposure the damage became widespread with proliferation of the interstitium around seminiferous tubules. Young and King (1986b) fed lower levels of zearalenone in the diet (0, 3, 6 and 9 mg zearalenone/kg feed) to boars from 32 days of age up to 145 or 312 days of age. Feeding up to 9 mg zearalenone/kg feed to the boars

did not affect the libido, but the boars fed the highest dose of zearalenone produced lower total and gel-free volumes of semen with lower total motile sperm. Zearalenone does not appear to affect mature boars. No adverse effects in reproductive parameters, including testicular size, libido, sperm motility and morphology, plasma testosterone and 17 $\beta$ -estradiol concentrations, were reported in mature Yorkshire boars fed increasing concentrations of purified zearalenone at 0, 2, 20 and 200 mg/kg ration for 8 weeks (Ruhr *et al.*, 1983).

The European Union (2006) guidance values for zearalenone in feedstuff (with a moisture content of 12%) for piglets and gilts is 0.1 mg/kg or ppm, for sows and fattening pigs is 0.25 mg/kg, and for calves, dairy cattle, sheep and goats is 0.5 mg/kg.

## Ruminants

Several case reports have associated dairy herd health problems and zearalenone in moldy feed. Young dairy heifers, 6–14 months of age, developed slight enlargement of at least one mammary gland quarter while fed moldy corn in a ration (Bloomquist *et al.*, 1982). Following a change in the ration, the heifers returned to normal 7 weeks later. Zearalenone contamination of the moldy ration was detected by thin-layer chromatography. Roine *et al.* (1971) reported turbid discharge from the vulva, obvious estrous behavior lasting for 1–2 weeks, and infertility in dairy cows and heifers. Strains of *F. graminearum* and *culmorum* were isolated from the feed that caused an increase in uterine weight in rats and were capable of producing between 3 and 9.5 mg zearalenone/kg feed. Ványi *et al.* (1974) reported a drop in milk production, feed intake and swelling of the vulva in dairy cows exposed to varying concentrations of zearalenone, ranging from 5 to 75 mg zearalenone/kg feed. In an experimental study, 18 cycling heifers were dosed with 0 or 250 mg of purified zearalenone daily through one non-breeding estrous cycle and the next two consecutive estrous cycles during which the heifers were bred (Weaver *et al.*, 1986a). The authors calculated that treated heifers were given an average of 250 mg zearalenone/364 kg body weight/day or 0.69 mg zearalenone/kg body weight/day. The control and treated heifers had conception rates of 87 and 62%, respectively, at a statistical probability of  $p < 0.065$ . Eighteen dairy cows (three cows per group) dosed orally with 0, 31.25, 62.5, 125, 250 and 500 mg of purified zearalenone daily for two consecutive estrous cycles had no changes in serum progesterone concentration, erythrocyte and leukocyte blood counts, packed cell volume, estrous cycle length, clinical health, or sexual behavior (Weaver *et al.*, 1986b).

Zearalenone can affect ewe reproduction when ewes are exposed to the mycotoxin prior to mating.

Zearalenone, administered orally at concentrations greater than 3mg/animal/day, given to ewes prior to mating depressed ovulation rates and reduced lambing percentages (Smith *et al.*, 1990). Ewes administered a similar range of oral doses of zearalenone (0, 1.5, 3, 6, 12 and 24mg/ewe/day) for 10 days, starting 5 days after mating, showed no effect of zearalenone exposure after mating on pregnancy rate or embryonic loss. Breeding rams fed a diet containing 12mg zearalenone/kg feed for 8 weeks had no significant adverse effects on semen volume, concentration, motility, or morphology during the trial and for 6 weeks after zearalenone feeding was ceased (Milano *et al.*, 1991).

## Equine

Gimeno and Quintanilla (1983) reported estrogenic signs of edematous vulvas, prolapsed vaginas, oversized uteruses and internal hemorrhage in mares, and severe flaccidity of genitals in two male horses fed corn screenings for 30 days in a field exposure. All sick animals collapsed with respiratory paralysis and sudden blindness, and died quickly. Analysis for zearalenone in the feed detected 2–3mg zearalenone/kg diet. While the authors tested for a variety of mycotoxins by thin-layer chromatography, fumonisins in the corn screenings were not determined because they had not been recognized as mycotoxins and a cause of death in horses. Fumonisin would have to be considered as a primary cause of some of the adverse effects, particularly blindness and death, in these horses.

In a study of six cycling trotter mares, Juhász *et al.* (2001) determined that daily oral administration of 7mg purified zearalenone starting 10 days after ovulation until the subsequent ovulation had no adverse effect on reproduction. Zearalenone had no effect on the length of the interovulatory intervals, luteal and follicular phases of the ovary, and did not significantly affect uterine edema. The authors noted that zearalenone exposure started 10 days after ovulation and the exposure period to zearalenone was short in this study. The dose of purified zearalenone represented a natural contamination of feed of about 1mg zearalenone/kg feed and ranged between 0.013 and 0.010mg zearalenone/kg body weight/day for approximately 8–10 days.

## Poultry

Growing female White Leghorn chickens dosed orally once with 15g zearalenone/kg body weight showed no adverse effects in reproductive tissues (Chi *et al.*, 1980). In a second experiment, chickens dosed orally or intramuscularly with increasing concentrations of zearalenone (0–800mg/kg body weight) for 7 consecutive

days had increased oviduct weights with increasing zearalenone doses. Poultry appear to be fairly resistant to the effects of zearalenone.

## TREATMENT

Quick removal of zearalenone-contaminated feed from the ration and replacement with clean feed is essential. Generally, 3–7 weeks following removal of the contaminated feed, animals will return to normal reproductive status. No zearalenone mycotoxin binder has been proven to be efficacious in any species of livestock by the U.S. Food and Drug Administration.

## CONCLUSIONS

Maintaining moisture concentrations less than 15–16% in feeds should adequately prevent zearalenone production during storage. When naturally contaminated feed must be used, incorporation into rations for less susceptible species, such as feedlot animals or poultry, should be considered. At naturally occurring concentrations of zearalenone in grains, residues in meat, milk and eggs are probably not significant.

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# Melamine and cyanuric acid

Karyn Bischoff

## INTRODUCTION

Melamine, or 1,3,5-triazine-2,4,6-triamine, is a small, nitrogen-rich molecule used in the manufacture of plastics, adhesives, cleaners and yellow dye. Though once considered practically non-toxic based on early laboratory animal studies, significant morbidity and mortality related to crystalluria, nephrolithiasis and nephrotoxicity have resulted from pet food contamination.

Beginning in early 2007, reports of renal failure in cats and dogs fed various commercial pet foods led to concerns about possible contamination, and a widespread recall of suspect foods was initiated prior to identification of the toxin. Eventually, >150 pet food products were identified as containing contaminated ingredients and were recalled. Analysis revealed that these products contained up to approximately 3200ppm melamine and 600ppm cyanuric acid (Cianciolo *et al.*, 2008; Skinner *et al.*, 2010). Samples of the imported wheat gluten contained 8.4% melamine as well as the following melamine breakdown products: 5.3% cyanuric acid, 2.3% ammelide and 1.7% ammeline (Rumbeiha *et al.*, 2010). Because melamine is 67% nitrogen based on the molecular weight, its addition to foodstuff increases the nitrogen content and thus the estimated protein content when protein is measured by common methods, such as the Kjeldahl method, which actually measure the nitrogen content.

It was later determined that similar episodes of pet food contamination occurred in Asia and South Africa. An incident in 2004 was estimated to have affected more than 6000 dogs and cats in the Republic of Korea,

Japan, Thailand, Malaysia, Singapore, Taiwan and the Philippines. Earlier in 2007, there was a melamine-associated pet food recall in South Africa (Osborne *et al.*, 2008; Yhee *et al.*, 2009). Aside from pet food, feeds for chickens, hogs and fish were also found to be contaminated (Reimschuessel *et al.*, 2008, 2009). Melamine-contaminated pet food scraps were fed on hog farms in seven U.S. states and contaminated feeds were traced to 38 poultry farms and at 197 fish hatcheries (Acheson, 2007; FDA, 2010). Investigation of renal failure in piglets in Spain between 2003 and 2006 found that the kidneys contained melamine, cyanuric acid and relatively high concentrations of ammelide and ammeline (Gonzalez *et al.*, 2009). Hundreds of fur-bearing raccoon dogs in China died after being fed melamine-contaminated feeds in 2008 (Bhalla *et al.*, 2009).

The 2007 pet food recall was considered a sentinel event by some (Osborne *et al.*, 2008; Lewin-Smith *et al.*, 2009). Indeed 1 year later melamine contamination of milk-based products, particularly baby formula, was detected in China. Chinese authorities detected melamine concentrations between 2.5 and 2563ppm in 13 commercial brands of milk powder and trace contamination in nine others (Bhalla *et al.*, 2009). Approximately 300,000 children could have been affected, more than 52,000 were hospitalized and six died. Children in Taiwan, Hong Kong and Macau could also have been affected (Hau *et al.*, 2009; Reimschuessel *et al.*, 2009; Skinner *et al.*, 2010). Due to global marketing of products and ingredients, melamine-contaminated products were found in almost 70 countries, including the United States.



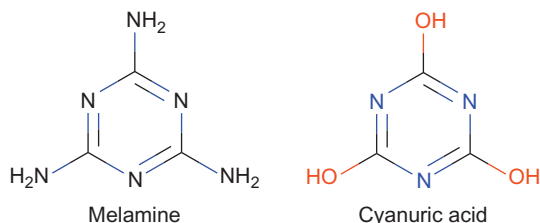


FIGURE 96.1 Structural formulas of melamine and cyanuric acid.

## SOURCE

Melamine, or 1,3,5-triazine-2,4,6-triamine (Figure 96.1), which contains 3 carbon atoms, 6 nitrogen atoms and 6 hydrogen atoms, was first synthesized in the 1830s and has found numerous uses in manufacturing. Melamine is polymerized with formaldehyde to produce a variety of durable resins, adhesives, cleansers and flame retardants. Melamine is also a major ingredient in the pigment Yellow 150, used in textile dyes and inks. China, the largest melamine producer, produces a quarter of the melamine used in the world.

Cyanuric acid, often found as a co-contaminant with melamine, is an intermediate produced during melamine manufacture and degradation. It is commonly used to stabilize chlorine in swimming pools and in the manufacture of bleach, disinfectant and herbicides (Dalal and Goldfarb, 2011).

## PHARMACOKINETICS/ TOXICOKINETICS

Melamine is minimally, if at all, metabolized in monogastrics, but could be partially metabolized in the rumen of cattle and small ruminants. Melamine does not accumulate over time in the animal body. Renal elimination of unchanged melamine is approximately 90% complete within 24 hours (Qin *et al.*, 2010). The half-life for urinary elimination of melamine is given as 6 hours in dogs (Lipschitz and Stokey, 1945). The elimination half-life in swine is approximately 4 hours (Yang *et al.*, 2011). Therefore, melamine should be almost completely excreted within 2 days of the last exposure in these monogastric species; however, crystals were seen microscopically in feline kidneys 8 weeks after dietary exposure to melamine and cyanuric acid (Cianciolo *et al.*, 2008). The elimination half-life of melamine in small ruminants is 3–6-fold slower than in monogastrics, possibly due to prolonged sequestration in the rumen

(Baynes *et al.*, 2010). Urinary excretion accounted for 54.1% of melamine administered to sheep, and 23.7% was found in the feces (Cruywagen *et al.*, 2011).

Detectable melamine concentrations have been reported in edible tissues from animals. It was estimated that 3.6% of melamine fed to sheep partitioned to muscle (Cruywagen *et al.*, 2011). Melamine concentrations in the kidney were higher than concentrations in the skeletal muscle or liver of lambs, and concentrations decreased below 20 ppb 4 days after cessation of exposure. Addition of cyanuric acid to the diet did not affect melamine deposition (Lv *et al.*, 2010). The highest tissue melamine concentrations in chickens fed melamine-containing diets were found in the kidneys, with lower concentrations in the liver and muscle. Tissue residues were depleted 10 to 20 days after exposure ceased (Bai *et al.*, 2010). Melamine administered to pregnant rats was detected in the placenta and fetuses (Jingbin *et al.*, 2010). However, reproductive and developmental effects have not been attributed to melamine in animal studies (Skinner *et al.*, 2010; Bischoff, 2011).

Melamine concentrations were detected in catfish and trout within 1 day of dosing, and the half-life of melamine in skeletal muscle of fish ranged from 1.5 to 4 days (Reimschuessel *et al.*, 2009). Due to the longer elimination half-life of melamine and cyanuric acid in fish, these compounds do not need to be given together. Melamine can be given 1 to 3 days after cyanuric acid (dependent on fish species) and produce crystals, and cyanuric acid can be given 7 to 14 days (dependent on species) after melamine and produce crystals (Reimschuessel *et al.*, 2010).

Melamine is excreted by dairy cattle into milk, particularly in high-producing cattle, though milk yield and composition are otherwise unaffected. Melamine can be detected in milk within 8 hours of exposure and remains detectable until 4 days after cessation of exposure. Transfer efficiency from feed to cows' milk was calculated to be between 0.66 and 0.95% and was not dependent on melamine dose (Shen *et al.*, 2010). Approximately 0.3% of a melamine dose was excreted in milk in dairy goats, and milk melamine concentrations remained above the level of concern (1.0 µg/mL) until 3 days after cessation of dosing (Baynes *et al.*, 2010).

Melamine fed to chickens is deposited within eggs within a day or 2 days post-exposure (Chen *et al.*, 2010; Bai *et al.*, 2010). The melamine concentration in eggs is proportional to the dietary concentration; a dietary concentration of 164 ppm could produce an actionable melamine concentration in eggs of 2.5 ppm (Chen *et al.*, 2010). A 4-day withdrawal period has been recommended for eggs from melamine-exposed chickens, though trace concentrations of melamine remain detectable in eggs for about 9 days after exposure ceases (Yang *et al.*, 2011).

## MECHANISM OF ACTION

Though previous animal studies found that both melamine and cyanuric acid were relatively nontoxic when given individually, they caused crystal formation in renal tubules when given together (Puschner and Reimschuessel, 2011). Melamine and cyanuric acid crystallize, forming a lattice structure at the molecular level, at a pH of 5.8 (Osborne *et al.*, 2008; Bhalla *et al.*, 2009). Crystals form in the distal convoluted tubules of the kidneys. Crystals recovered from kidneys and urine of cats that ingested melamine-contaminated food contained 70% cyanuric acid and 30% melamine based on infrared spectra results (Osborne *et al.*, 2008; Thompson *et al.*, 2008).

Renal pathology is believed to result from intratubular obstruction causing increased intrarenal pressure. This may be the explanation of the renal hemorrhage and inflammation reported in cats and dogs. Since melamine has diuretic properties, pre-renal azotemia could contribute to the renal pathology (Bhalla *et al.*, 2009).

Cyanuric acid did not contribute to the formation of urinary calculi in children as it did in companion animals (Gao *et al.*, 2010). However, calculi in children were produced by a similar interaction between melamine and uric acid. This interaction has also been reported in poultry (Bai *et al.*, 2010). Humans and most other primates lack the enzyme uricase, which converts uric acid to allantoin (Reimschuessel *et al.*, 2008). Compared to adults, human infants excrete between 5 and 8 times as much uric acid, which may increase their susceptibility to melamine toxicosis (Skinner *et al.*, 2010). Urinary pH <5.5 is associated with the formation of urate crystals and low pH is believed to be involved in melamine/urate crystal formation (Gao *et al.*, 2010). Crystals associated with nephrotoxicosis in these infants contained melamine and uric acid at a molar ratio of 1:1–2, respectively (Skinner *et al.*, 2010; Wen *et al.*, 2010). Birds and reptiles also excrete uric acid; therefore, susceptibility in these species could be similar to those seen in human neonates. Dalmatian dogs, too, have reduced uric acid metabolism, and thus excrete uric acid.

## TOXICITY

Melamine has relatively low toxicity based on animal studies. The oral LD<sub>50</sub> of melamine is 3200mg/kg in male rats, 3800g/kg in female rats, 3300mg/kg in male mice and 7000mg/kg in female mice. Long-term administration of melamine to laboratory rodents at concentrations ranging from 0.225 to 0.9% of the diet produces urolithiasis. Lesions in the urinary bladder, including transitional cell carcinoma, were observed in rats fed diets containing

0.45% melamine (Melnick *et al.*, 1984). The mechanism of carcinogenesis was most likely secondary to epithelial hyperplasia caused by mechanical irritation (Melnick *et al.*, 1984; Hau *et al.*, 2009). Urolithiasis was consistently identified in rats fed diets containing 0.3 to 3.0% melamine (Okumura *et al.*, 1992). Renal cortical fibrosis and lymphoplasmacytic nephritis were reported in female rats fed diets containing 0.45% melamine (Melnick *et al.*, 1984). Among domestic animals, sheep given single (217mg/kg) or multiple (200 to 1351mg/kg/d) doses of melamine had dose-dependent clinical signs of anorexia, anuria and uremia after 5 to 31 days (Clark, 1966). Dogs fed 125mg/kg melamine had crystalluria but no other adverse effects were identified (Lipschitz and Stokey, 1945).

Cyanuric acid has similarly low toxicity when given alone. It produces degenerative renal changes in guinea pigs when given at doses of 30mg/kg body weight for 6 months, in rats fed a diet containing 8% monosodium cyanurate for 20 weeks, and in dogs fed a diet containing 8% monosodium cyanurate. Lesions included ectasia of the distal collecting tubules and multifocal epithelial cell proliferation (Canelli, 1974).

The combination of melamine and cyanuric acid is markedly more toxic to most domestic animals than either compound when given alone. Administration of 50mg/kg melamine or cyanuric acid to rats did not result in adverse effects, but when both were given together at the dose of 50mg/kg, evidence of nephropathy was observed (Puschner and Reimschuessel, 2011). Cats fed diets containing up to 1% melamine or cyanuric acid alone had no evidence of clinical abnormalities. However, cats fed diets containing 0.2% each of melamine and cyanuric acid had evidence of acute renal failure and crystaluria within 48 hours due to renal tubular necrosis. Lesions typical of those associated with the pet food recall were observed (Puschner *et al.*, 2007). A pig fed 400mg/kg melamine and 400mg/kg cyanuric acid daily had bloody diarrhea within 24 hours, which resolved. Necropsy revealed perirenal edema and round golden-brown crystals with radiating striations in the kidneys. Similar lesions were present in tilapia, rainbow trout and catfish dosed with 400mg/kg each of melamine and cyanuric acid per day for 3 days, though most of the fish survived the renal damage (Reimschuessel *et al.*, 2008).

A recent study suggested that melamine is neurotoxic as it impaired synaptic plasticity in hippocampus and thereby learning and memory deficits in rats (An *et al.*, 2011).

## CLINICAL SIGNS

Early clinical signs of vomiting and inappetance were apparent in cats and dogs ingesting contaminated food, followed later by evidence of renal failure. The most

common clinical signs in cats and dogs were inappetence, vomiting, polyuria, polydipsia and lethargy (Cianciolo *et al.*, 2008). The most common symptoms described in children were increased or reduced frequency of urination or anuria, hematuria, stranguria and unexplained crying, but many children were asymptomatic (Hau *et al.*, 2010; Wen *et al.*, 2010; Hu *et al.*, 2010).

## CLINICAL PATHOLOGY

Affected cats had urine specific gravities  $<1.035$  and elevated serum urea nitrogen and creatinine concentrations within 36 hours of exposure, hyperkalemia and hyperphosphatemia (Cianciolo *et al.*, 2008; Puschner and Reimschuessel, 2011). Urinalysis revealed the presence of circular green-brown crystals in urine sediment (Cianciolo *et al.*, 2008). Serum chemistry findings included elevated serum urea nitrogen and creatinine concentrations.

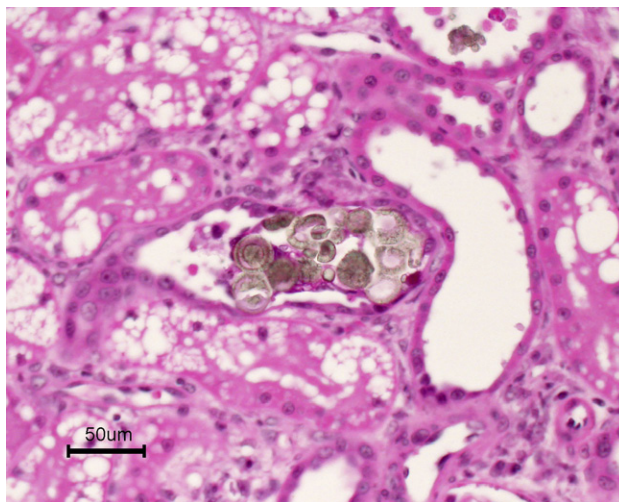
Serum chemistry findings in affected infants included elevated serum potassium, urea nitrogen and creatinine concentrations (Sun *et al.*, 2010). Hematuria and the presence of fan-shaped crystals were reported in urine samples and urine pH ranged from 5.0 to 7.5 (Hau *et al.*, 2009).

## PATHOLOGY

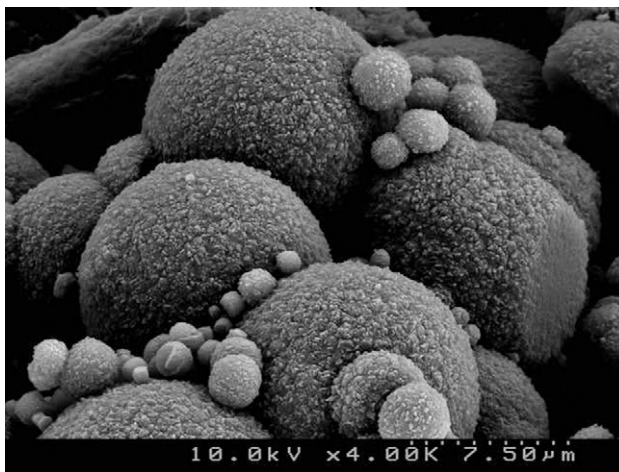
Post-mortem findings from animals that died typically included bilateral renomegaly and evidence of uremia (Thompson *et al.*, 2008; Cianciolo *et al.*, 2008).

Microscopic lesions were found primarily in the kidneys. Renal tubular necrosis with evidence of rupture and regeneration were present. Distal convoluted tubules contained large golden-brown birefringent crystals (15 to 80 micrometers in diameter; Figure 96.2) with centrally radiating striations, sometimes in concentric rings, and smaller amorphous crystals (Cianciolo *et al.*, 2008; Thompson *et al.*, 2008). Crystals were spherical with a hedgehog-like appearance on images from scanning electron microscopy (Figure 96.3). Crystals were still present in distal convoluted tubules 8 weeks after cessation of exposure to contaminated feed (Cianciolo *et al.*, 2008). Perivascular inflammation of subcapsular veins was also reported.

Melamine/cyanuric acid crystals could be differentiated from more common types of urinary crystals based on morphology and histochemistry or special staining. Von Kossa stains calcium oxalate and calcium phosphate crystals and Alizarin red S at pH between 4.1 and 4.3 stains calcium phosphate crystals only, but neither stains melamine/cyanuric acid crystals. Oil red O, usually



**FIGURE 96.2** Renal histology from a cat that ingested melamine and cyanuric acid contaminated food. Typical crystals are present within the renal tubule at the center. (Courtesy of Dr. R. Cianciolo).



**FIGURE 96.3** Scanning electron micrograph of melamine/cyanuric acid crystals from the urine of a cat. (Courtesy of Dr. B. Hoff.)

used to identify lipids, stains melamine/cyanuric acid crystals but not calcium oxalate or calcium phosphate crystals (Cianciolo *et al.*, 2008). Melamine/cyanuric acid crystals dissolve over time when kidney is stored in formalin (Puschner and Reimschuessel, 2011).

A cat that was euthanized 8 weeks after exposure had moderate tubular necrosis with regeneration and severe crystalluria. The finding of severe perivenous fibrosis and venous luminal stenosis in this cat suggested that cats that survived acute renal failure may have been predisposed to chronic renal ischemia leading to chronic renal disease (Cianciolo *et al.*, 2008).

Lesions secondary to uremia were sometimes observed in affected cats and included bilateral white



plaques on the ventral surface of the tongue and gastric mineralization (Cianciolo *et al.*, 2008).

A renal biopsy from an affected child had glomerular sclerosis, swelling and necrosis of renal tubular epithelium, tubular dilation with the presence of material consistent with a crystal, and an interstitial lymphoplasmacytic infiltrate. These changes were hypothesized to be due to urinary tract obstruction (Sun *et al.*, 2010). Stones in the urinary tract ranged in size from 2 to 18 mm in diameter, and approximately half were <5 mm in diameter. Diameter of the stone was dependent on the concentration of melamine in the diet, but not on the duration of exposure (Hu *et al.*, 2010). Renal calculi were usually bilateral, and calculi were also found in the ureter, unilaterally or bilaterally, and in the urinary bladder (Hau *et al.*, 2009; Gao *et al.*, 2010; Wen *et al.*, 2010).

## DIAGNOSIS

Diagnosis of melamine poisoning is based on the presence of melamine and cyanuric acid in samples of urine or kidney from affected animals, or contaminated food or feed. Gas chromatograph/mass spectroscopy (GC/MS) and liquid chromatography/tandem MS are used to analyze for melamine and cyanuric acid (Cianciolo *et al.*, 2008; Hon *et al.*, 2011; Puschner and Reimschuessel, 2011). Infrared spectroscopy or Raman spectrometry can be used to determine the composition of the urine crystals (Puschner and Reimschuessel, 2011). Identification of crystals on histopathology or urinalysis is also helpful in the diagnosis.

## TREATMENT AND PROGNOSIS

The basic treatment regimens for crystalluria and urolithiasis related to melamine ingestion include fluid therapy and supportive care in both veterinary and pediatric patients (Anonymous, 2007; Wen *et al.*, 2010). Increased water intake and fluid therapy were used to increase urine output. Because low urinary pH is associated with crystal formation in infants, alkalization of the urine was used to maintain urine pH between 6.0 and 7.8 in affected children. Sodium bicarbonate or potassium citrate was added to intravenous fluids for this purpose (Gao *et al.*, 2010; Wen *et al.*, 2010).

Antispasmodic drugs such as anisodamine or atropine were given to facilitate excretion of uroliths in children, and pain management was instituted (Bhalla *et al.*, 2009; Wen *et al.*, 2010). Most children recovered with this conservative management (Gao *et al.*, 2010; Wen *et al.*, 2010).

However, hemodialysis was required in some patients, as was surgical intervention (Wen *et al.*, 2010). Most children recovered fully, but 12% were found to have renal abnormalities 6 months after treatment (Liu *et al.*, 2010).

Analysis of 586 cases reported during the pet food recall of 2007 by veterinarians and accredited veterinary diagnostic laboratories found 451 cases matching the case definition of melamine toxicosis, 65.5% were cats and 34.4% were dogs. Older animals and animals with preexisting conditions were less likely to survive, and 73.3% of affected dogs and 61.5% of affected cats died (Rumbeiha *et al.*, 2010). However, >80% of exposed cats during the original feeding trials survived, suggesting that not all exposed cats become severely affected (Cianciolo *et al.*, 2008).

## CONCLUSIONS

Melamine poisoning in small animals and infants was associated with intentional contamination of pet food and milk products to increase the nitrogen concentration, and therefore the apparent protein concentration. Although there are some data on the number of infants affected, there is no way to determine the number of cats and dogs affected by the pet food recalls in 2007 because of a lack of veterinary care for many animals and lack of central reporting of morbidity and mortality for companion animals. Estimates vary depending on the source and have ranged from 571 to 39,000 cats and dogs (Osborne *et al.*, 2008; Puschner and Reimschuessel, 2011). Although many cats and dogs succumbed to melamine toxicosis, others survived with supportive and symptomatic care. There are little data on the long-term effects of melamine on cats and dogs.

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# Ionophores

Meliton N. Novilla

## INTRODUCTION

Ionophores are compounds that form lipid soluble, dynamically reversible complexes with cations and by this means facilitate specific ionic transport across biologic membranes (Pressman, 1976; Reed, 1982; Taylor *et al.*, 1982). There are two major subclasses of ionophores, (1) neutral ionophores, which are highly toxic because they form charged complexes that are capable of perturbing biologic membranes and action potentials, and (2) carboxylic ionophores, which form zwitterionic complexes with cations and promote electrically neutral cation exchange diffusion that is tolerated better in intact organisms. The ionophoric activity may alter normal concentration gradients resulting in cellular ion imbalance, pH change, calcium overload, lipid peroxidation and disruption of plasma membranes. The alteration in the membrane transport of ions is the basis for the metabolic, organic and functional effects of this class of compounds. Since their pharmacologic activity is dose related, the usefulness of carboxylic ionophores is based on selective toxicity to protozoan parasites and bacteria and margins of safety in the approved target species. Desirable effects occur when animals are provided feed containing approved dosage ranges, but higher levels may result in adverse effects.

This chapter attempts to provide an overview of ionophores as they relate to veterinary medicine, with emphasis on ionophore-induced toxicity. Large numbers of ionophore safety and toxicity studies have been conducted in support of marketing approval, but results of many studies are unpublished. For this reason, information presented was gleaned from published laboratory and field reports of toxicoses in various species of animals, available reviews and Freedom of Information

summaries obtained from the U.S. FDA. However, due to space limitations, not all information can be included, hence apologies are extended to laboratory scientists and field researchers whose work has been omitted or inadvertently missed.

## BACKGROUND

Presently, seven carboxylic ionophores are approved for the control of coccidiosis and promotion of growth and feed efficiency in several animals of economic importance. Since their introduction carboxylic ionophores have played significant roles in livestock and poultry production systems throughout the world. Monensin, first introduced as Coban<sup>®</sup> in the United States for the control of coccidiosis in chickens in 1971, was later marketed in 1975 as Rumensin<sup>®</sup> to promote growth and/or increase feed efficiency in cattle. Similarly, lasalocid has been marketed since 1977 as Avatec<sup>®</sup> for chickens and since 1982 as Bovatec<sup>®</sup> for cattle. Salinomycin, narasin and maduramicin were approved for chickens, respectively in 1983, 1986 and 1989, while laidlomycin and semduramicin were approved in 1994 for use in cattle and chickens, respectively. (See [Figure 97.1](#).)

Other benefits of ionophore use include (1) reduction of coccidial oocyst discharge in ruminants, (2) prevention of acute bovine pulmonary edema and emphysema, (3) decreased incidence of bloat, (4) prevention of ruminal lactic acidosis, and (5) amelioration of ketosis in lactating dairy cows (Novilla, 2004; Duffield *et al.*, 2002; Heuer *et al.*, 2001; Parker *et al.*, 1986; Nocerini *et al.*, 1985; Potter *et al.*, 1984). The reduction of deaths in some cattle

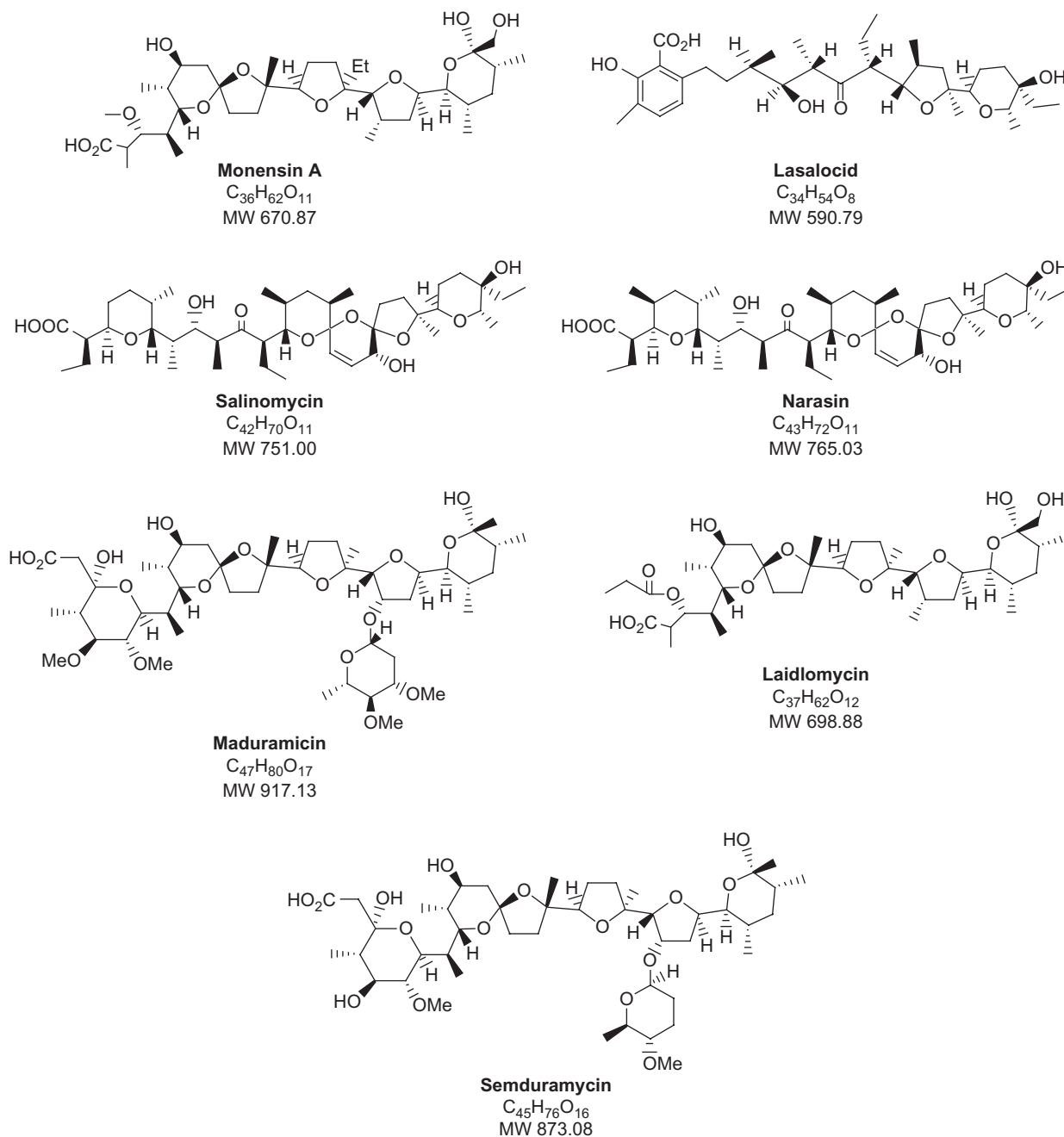


FIGURE 97.1 Structures of carboxylic ionophores.

herds has been hypothetically related to the reduction of indigestion, metabolic stress, bloat and enterotoxemia associated with monensin feeding.

Potential uses of the ionophores are under experimental investigation in many parts of the world and off-label uses of some ionophores are known to occur. Monensin has been used for the control of toxoplasmosis in pregnant sheep (Buxton *et al.*, 1988), disseminated visceral coccidiosis in cranes (Carpenter and Novilla, 1992), diarrheal disease in swine (Kyriakis *et al.*, 1993), gregarine infections in shrimp (Jones *et al.*, 1994; Fajer-Avila

*et al.*, 2005) and microsporidial gill disease in fish (Becker *et al.*, 2002). The use of monensin to improve efficiency of milk production in dairy cattle has been approved in the U.S. (FDA, 2004). Experimental studies on the use of ionophores for the treatment of malaria (Adovelande and Schrevel, 1996; Gumila *et al.*, 1997), mycobacteriosis (Brumbaugh *et al.*, 2004; Greenstein *et al.*, 2009), lead poisoning (Hamidinia *et al.*, 2002) and as an aid in the potentiation of anti-cancer therapies (Griffin *et al.*, 1993; Shaik *et al.*, 2001) have been conducted. These studies were conducted in cell culture or as challenge studies

TABLE 97.1 General pharmacology studies on monensin

Test system	Species	Monensin dose (mg/kg)	Route	NOEL (mg/kg)
General behavior	Mouse	0, 10 and 30	Oral	10
Coordinating activity of skeletal muscles	Mouse	0, 10 and 30	Oral	10
Anti-electroshock seizures	Mouse	0 and 30	Oral	30
Analgesic effect	Mouse	0, 10 and 30	Oral	10
Spinal reflex	Cat	0 and 30	Oral	30
Electroencephalogram (d-tubocurarine immobilized)	Cat	0 and 30	Oral	30
Circulatory, respiratory and autonomic effects	Cat	0 and 30	Oral	30
Charcoal meal transit	Mouse	0, 10 and 30	Oral	10
Gastric secretion	Rat	0 and 30	Oral	30
Isolated ileum	Guinea pig	$1 \times 10^{-5}$ g/mL	<i>In vitro</i>	$1 \times 10^{-5}$ g/mL
Carragenin-induced edema	Rat	0 and 30	Oral	30
Cardiovascular study	Dog (conscious)	0, 0.0069, 0.0138, 0.0345, 0.0690 and 0.138	Intravenous	0.0345
		0, 0.138, 0.345, 0.690 and 1.38	Oral	0.345 <sup>a</sup>
Cardiovascular and respiratory effects	Dog (anesthetized)	0, 0.00069, 0.0014, 0.0035, 0.0069, 0.014, 0.035, 0.069, 0.14, 0.35, 0.69 and 1.4	Intravenous	0.0035
Cardiovascular and respiratory effects	Pig (anesthetized)	0, 0.00069, 0.0014, 0.0035, 0.0069, 0.014, 0.035, 0.069, 0.14, 0.35 and 0.69	Intravenous	0.0035

<sup>a</sup>Based on transient increases in coronary blood flow at doses  $\geq 0.69$  mg/kg.

in laboratory animals and have shown some degree of efficacy. In spite of these encouraging results, there have been no reports of investigational use of ionophores in humans.

## PHARMACOLOGY/ PHARMACOKINETICS

### Pharmacology

Investigational studies were conducted to determine if ionophores had any undesirable pharmacological properties. A series of general pharmacology studies was conducted with monensin (Novilla, 2004). Earlier, Hanley and Slack (1982) reviewed the pharmacology of lasalocid. The studies with monensin assessed its effects on the central, peripheral and autonomic nervous systems and the digestive, respiratory and cardiovascular systems. The test systems with doses and route of administration and no-observed-effect-level (NOEL) are shown in Table 97.1.

In mice, an oral dose of 10 mg monensin/kg produced no significant effects on general behaviors, coordinating activity in skeletal muscles, electroshock seizures and acetic acid writhing. However, slight sedation, decreased sensitivity to tactile stimulation, slight depression of muscular coordination and depressed acetic acid writhing occurred at 30 mg/kg. In cats, immobilized by d-tubocurarine or anaesthetized with urethane and  $\alpha$ -chloralose, an oral dose of 30 mg/kg produced no effect on the electroencephalogram and the spinal

reflex. At the same dose in anaesthetized cats, the heart rate, electrocardiogram, respiration and blood pressure responses to epinephrine, acetylcholine, histamine and contractions of the nictitating membrane in response to electrical stimulation of the cervical sympathetic ganglion were not affected. Charcoal meal transit in mice was not affected by an oral dose of 10 mg/kg while 30 mg/kg significantly depressed the transit rate. Gastric secretion was not affected by an oral dose of 30 mg/kg in rats. In isolated guinea pig illegal preparations, monensin in a bath concentration of  $10^{-5}$  g/mL had no antagonistic effects on contractures induced by acetylcholine, histamine and barium chloride. In the rat anti-carrageenan edema test, monensin given orally at 30 mg/kg did not significantly inhibit carrageenan-induced edema of the hind paw.

The studies targeting specific organ systems of laboratory animals indicated that monensin at a dose of 10 mg/kg orally produced no effect on the central, peripheral and autonomic nervous systems or the respiratory and digestive systems. However, striated (heart and skeletal) muscle has been identified as the primary targets of toxicity in laboratory animals and domestic livestock given large doses of monensin and other ionophores by oral or parenteral routes (Todd *et al.*, 1984; Novilla and Folkerts, 1986; Van Vleet *et al.*, 1991; Novilla and Todd, 1991; Dowling, 1992).

The cardiovascular effects of oral and intravenous administration of monensin were evaluated in conscious dogs (Holland, 1978). In the oral study, doses of 0, 0.138, 0.345, 0.69 or 1.38 mg monensin/kg body weight in 15 mL of 10% acacia were given by gavage to four or six dogs. Effects were limited to increased coronary



blood flow at doses  $\geq 0.69$  mg monensin/kg. At the 0.69 mg/kg dose, coronary flow increased from 24 mL/minute before dosing to a maximum of 40 mL/minute 17.5 minutes after dosing. At the 1.38 mg/kg dose, coronary flow increased from 18 mL/minute pre-dosing to a maximum of 49 mL/minute 13 minutes after dosing. In both cases, coronary blood flow had returned to normal levels by 30 minutes after dosing. Heart rate and mean blood pressure of monensin-treated dogs did not change significantly from the controls at any dose level. Coronary artery flow did not increase following doses of 0.138 and 0.345 mg monensin/kg. In the intravenous (IV) study, coronary blood flow, blood pressure and heart rate were measured in each of six dogs given total cumulative doses of 0.0069, 0.0138, 0.0345, 0.069 and 0.138 mg monensin/kg body weight. Four control dogs were given dextrose alone. Coronary artery flow significantly increased at 0.069 and 0.138 mg/kg and mean blood pressure increased significantly at 0.138 mg/kg. Coronary artery flow did not change significantly following doses  $\leq 0.0345$  mg/kg while heart rate did not change significantly from the controls at any dose level. Mean blood pressure also did not change significantly at dose levels of 0.0069, 0.0345 and 0.069 mg/kg. Based on the transient increases in coronary blood flow observed in these studies, the no observed effect levels (NOELs) were 0.345 and 0.0345 mg monensin/kg following oral and intravenous dosing, respectively.

The cardiovascular effects of monensin are similar to those observed for lasalocid. Hypothetically, based on their inotropic and chronotropic properties, ionophores were considered as potential therapies for human cardiac disease (Pressman, 1976; Hanley and Slack, 1982).

These pharmacology studies are considered relevant to the overall safety of ionophores as they provide insight into the secondary pharmacological actions and support the margins of safety for exposure in humans during the manufacturing processes or in the mixing or handling of the premixes in preparing feeds for the target species. In regard to the dog cardiovascular studies, monensin-induced increases in coronary blood flow observed at oral doses of 0.69 and 1.38 mg/kg were transient and considered not to be toxicologically important. At these doses, the respective increases of 67 and 172% in blood flow are in the physiologic range by virtue of the autoregulatory coronary flow reserve. Further, the phenomenon was not considered to be deleterious because recognized indicators of cardiovascular toxicity, such as increased blood pressure and/or heart rate, were not observed concurrently in the oral study. If it can be assumed that the relative potency of IV dosing to produce increased blood pressure is 11 times greater than by oral dosing (Holland, 1978), then the equivalent dose of 1.52 mg monensin/kg given orally by capsule would be expected to produce this change. According to Pressman

and Fahim (1983), a single oral dose of 2.0 mg/kg in dogs produced increased heart rate and arterial pressure for up to 2 hours after dosing, hence the lower dose of 1.38 mg monensin/kg may be considered as the no observed adverse effect level (NOAEL). If one takes 0.69 mg monensin/kg as a pharmacologically significant oral dose and 0.345 mg/kg as a NOEL for humans, then a 60 kg person would require 41.4 mg of monensin to manifest a transient effect on coronary blood flow while 20.7 mg monensin will have no effect (Donoho, 1984).

Human exposure to systemic levels of monensin and other ionophores high enough to produce any toxicological effects is very unlikely. With the use of safety equipment and close adherence to good manufacturing practices (GMPs) and label instructions, personnel manufacturing and handling ionophore products should not be subjected to undue risk. Furthermore, it is highly unlikely for these exposures to occur in humans consuming meat and milk products from animals fed a complete feed formulated with ionophores because residues are extremely low (Novilla, 2004).

## Pharmacokinetics

Tissue residue and metabolism studies were required to be conducted on all marketed ionophores to support human safety. For monensin, several studies with both  $^{14}\text{C}$ -monensin and unlabeled compound have shown that following oral administration in cattle and other species, monensin is rapidly absorbed and extensively metabolized by the liver and that most of the administered monensin and its metabolites are excreted in the bile. Results of biochemical studies on the metabolic fate of monensin in animals have been published (Davison, 1984; Donoho, 1984; Atef *et al.*, 1993; Nebbia *et al.*, 2001). The metabolite pattern for monensin is similar in cattle, chickens, rats, turkeys, sheep and pigs. Metabolite characterization studies indicated that hydroxylation (*o*-demethylation and oxidation) is the primary metabolic pathway of monensin resulting in low concentrations of a large number of polar metabolites in the feces of  $^{14}\text{C}$ -monensin-dosed animals. The pattern of metabolites in cattle and rats is similar. Based on radiolabeled studies in steers and dairy cows, the liver had the highest total monensin residue following zero withdrawal (Davison, 1984; Donoho, 1984; Kennington *et al.*, 1995). Parent monensin and five metabolites were identified in liver, bile and/or feces from monensin-treated steers and dairy cows. Studies have shown that metabolite M-1 (*o*-desmethyl monensin) was 20 times less active than parent monensin, the marker residue. Hence, the suspected first step in monensin metabolism appears to eliminate most of the biological activity. Plasma levels of monensin are low and decline rapidly in cattle treated with monensin. In cattle administered

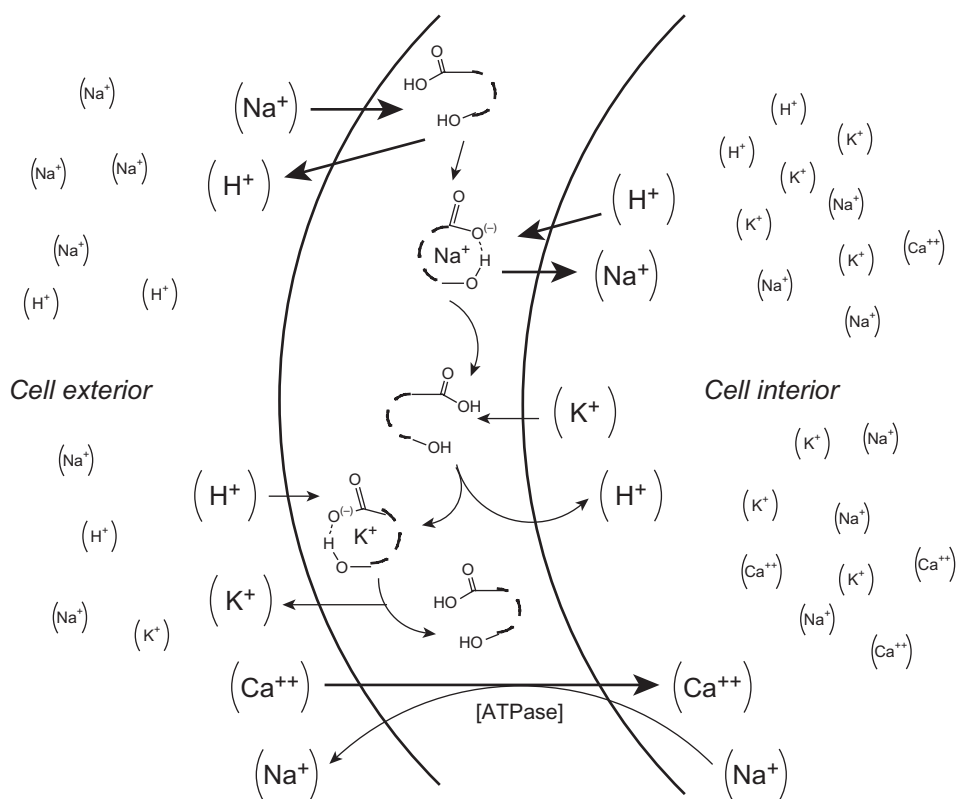
$^{14}\text{C}$ -monensin, residues of radioactivity depleted rapidly from the tissues. In a recent tissue residue study, no monensin was detected in extracted fat, liver, kidney and muscle tissues from cattle given feed containing 100 ppm to provide a dose of 0.9 mg monensin/kg body weight/day for 28 days (Coyle and Walker, 2005). Feeding of muscle, liver and other viscera from cattle provided dietary levels of 165 ppm monensin (five times the approved use level) to rats and dogs for 3 months produced no adverse effects. Moreover, monensin does not accumulate in tissues of animals even when given intoxicating doses (Donoho, 1984; Atef *et al.*, 1993). Thus, exposure of humans to residues of monensin in meat or milk from animals treated according to the label instructions will be very limited and will be due primarily to the parent compound. Furthermore, when residues, primarily the parent compound, are present in animal products intended for human consumption, they will be at minimal levels for which a sufficient margin of safety has been demonstrated in the toxicology studies (Novilla, 2004).

Pharmacokinetic studies with other marketed ionophores generated data similar to that of monensin. Results of these studies in the FOI summaries facilitated determination of the safe residue concentration, acceptable daily intake, marker residue and tolerance, and withdrawal time by the U.S. FDA.

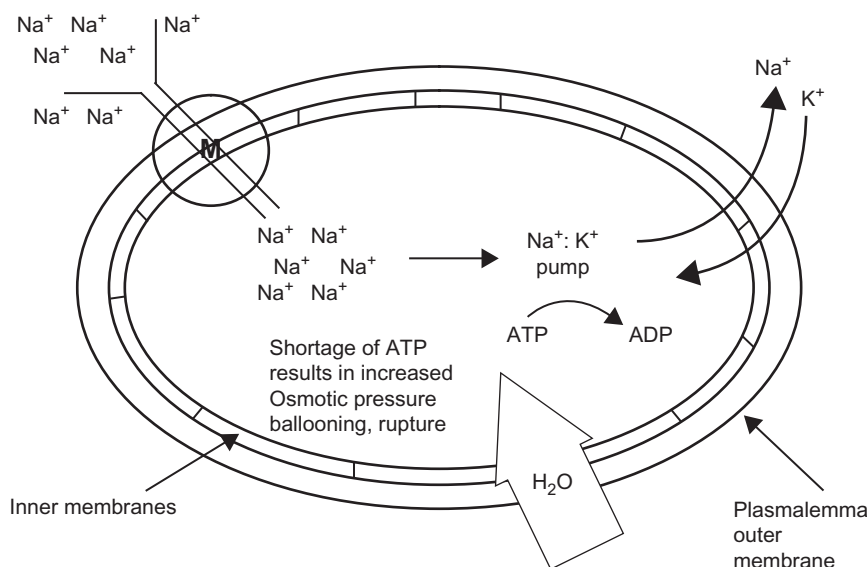
## MECHANISM OF ACTION

Carboxylic ionophores are known to form cationic ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ ) complexes that enhance their transport across bimolecular lipid membranes (Pressman, 1976; Pressman and Fahim, 1982, 1983; Taylor *et al.*, 1982; Reed, 1982; Mollenhauer *et al.*, 1990). However, ion proclivities differ and evidence gathered in various laboratories indicates that carboxylic ionophores may have multiple effects at the cellular level. (See Figure 97.2.)

In this diagram, monensin, classified as an  $\text{Na}^+$  selective ionophore, binds to  $\text{Na}^+$  outside the cell, carries it into the cell and produces higher intracellular concentrations of  $\text{Na}^+$ . Influx of  $\text{Na}^+$  is counterbalanced by an efflux of  $\text{K}^+$  but, since  $\text{K}^+$  efflux is slow, an initial efflux of proton ( $\text{H}^+$ ) occurs, which may result in intracellular alkalosis. With salinomycin and narasin and others that show  $\text{K}^+$  selectivity, the  $\text{K}^+$  egress is countered by an initial  $\text{H}^+$  ingress, which could result in intracellular acidification. Salinomycin and narasin preferentially complex  $\text{K}^+$  over  $\text{Na}^+$  at a ratio of 4:1. An ionophore-mediated rise in intracellular  $\text{Na}^+$  is known to increase the intracellular levels of  $\text{Ca}^{++}$  due to an ATPase-driven exchange mechanism at the cell membrane. Monensin was reported to shorten the duration of the action



**FIGURE 97.2** Diagram of cation ion exchange diffusion across plasma membranes facilitated by monensin. Large arrows indicate major transport activity.



**FIGURE 97.3** Effect of ionophores on ion and water dynamics in the coccidia. M = monensin. From [Thomas et al. \(1985\)](#) Elanco Animal Health.

potential and suppressed the pacemaker potential in cardiac tissue ([Novilla, 2004](#)). These membrane current effects were related to transmembrane alterations in the gradients of  $\text{Na}^+$  and  $\text{K}^+$  ions and to increased intracellular  $\text{Ca}^{++}$  following the increase in cytoplasmic  $\text{Na}^+$  concentration, probably via an  $\text{Na}^+/\text{Ca}^{++}$  exchange mechanism. Lasalocid directly translocates  $\text{Ca}^{++}$  because it forms complexes with divalent cations with a range of complexing and transport capabilities including primary amines, e.g., catecholamines ([Hanley and Slack, 1982](#)). Monensin also causes release of catecholamines from cultured adrenal chromaffin cells, and salinomycin causes a multifold augmentation of plasma catecholamines in animals. Catecholamines and toxic oxidation products have been implicated in myocardial necrosis through greater influx of  $\text{Ca}^{++}$  and formation of free radicals.

By special immunohistochemistry staining, monensin treatment was shown to increase  $\text{Na}^+\text{K}^+$ -ATPase and  $\text{Ca}^{++}$ -ATPase and NO synthase activities in the heart of chickens ([Calo et al., 2002, 2003](#)). Nitric oxide, the product of NO synthases, is recognized as a regulator of calcium homeostasis. Alterations in the concentrations of  $\text{Ca}^{++}$  and other cations extracellularly as well as changes in their intracellular distribution have been associated with changes in subcellular organelles and cell damage ([Shier and DuBourdieu, 1992; Calo et al., 2002, 2003; Sandercock and Mitchell, 2004](#)).

Carboxylic ionophores directly affect the asexual and sexual developmental stages of *Eimerian* coccidia by causing the normal transport of  $\text{Na}^+$  and  $\text{K}^+$  ions to fail ([Smith and Galloway, 1983](#)). In studies with free *Eimerian* sporozoites, monensin causes increased  $\text{Na}^+$  levels in the parasite, increased activity of the sodium pump,

decreased intra-sporozoite ATP and stimulation of glycolysis ([Smith et al., 1981](#)). Continuous exposure to monensin results in an increased osmotic gradient within the developing sporozoan parasite. Water follows and the organisms utilize energy to maintain intracellular homeostasis. When energy is exhausted the parasite swells with damage to organelles and death ensues ([Smith et al., 1981; Mehlhorn et al., 1983](#)). While most ionophores target free coccidial stages in the intestinal lumen (sporozoites and merozoites), the first intracellular generation (trophozoites) was most sensitive to lasalocid ([Long and Jeffers, 1982](#)). (See [Figure 97.3](#).)

Similar events occur in susceptible bacteria. By reducing acetic and butyric acid producing (Gram-positive) bacteria and promoting growth of propionic acid producing (Gram-negative) bacteria, ionophores improve the efficiency of rumen fermentation resulting in a greater retention of feed energy in metabolizable nutrients ([Bergen and Bates, 1984; Russell and Strobel, 1989](#)). Monensin, lasalocid and laidlomycin have been shown to alter the molar ratios of volatile fatty acids (VFAs) such that there is increased propionate and decreased acetate and butyrate without altering total VFA concentration.

## TOXICITY

### Pathogenesis

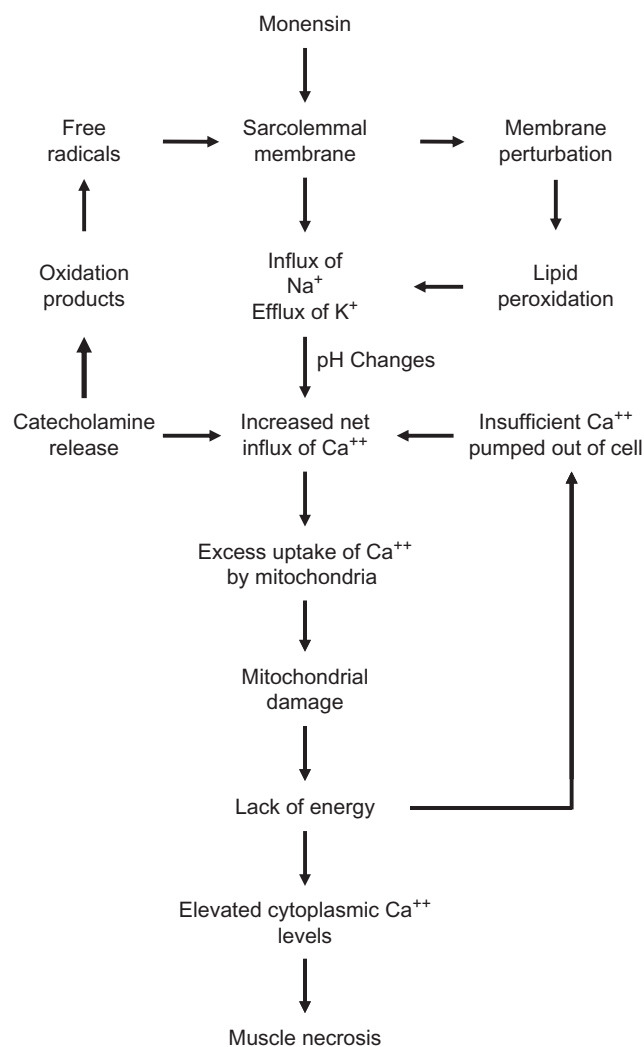
The exact mechanism of the toxicity induced by ionophores is not known. However, reasonable hypotheses have been generated based on their inherent ionophoric

activity (Van Vleet *et al.*, 1983c, 1991; Novilla and Folkerts, 1986). All ionophores facilitate transmembrane ion fluxes and dissipation of ion gradients, which are exaggerated at toxic levels. Cells respond to the metabolic insult by expending energy to maintain homeostasis. When homeostatic mechanisms are exceeded, toxicity ensues from excessive influxes of  $\text{Na}^+$  and  $\text{Ca}^{++}$  leading to degeneration and necrosis of cardiac and skeletal muscle cells. Although the ionophore mode of action is simple, four biochemical changes, including intracellular pH effects, calcium overloading, catecholamine release and lipid peroxidation, probably occur during ionophore toxicoses. (See Figure 97.4.)

In the diagram, monensin facilitates cation exchange diffusion as it intercalates with plasma membranes (Pressman, 1976; Reed, 1982). The exaggerated pharmacologic activity at toxic levels disrupts not only osmotic gradients but the intracellular pH as well. Since drastic changes in acid-base balance are incompatible with life, the pH shifts may be responsible for the peracute deaths observed with very high toxic levels of ionophores (Novilla and Folkerts, 1986). The monensin-induced entry of  $\text{Na}^+$  is followed by entry of calcium, due to an ATPase-driven exchange mechanism at the cell membrane. A calcium ionophore like lasalocid or A23817 promotes  $\text{Ca}^{++}$  entry directly. Ionophores are known to trigger the release of  $\text{Ca}^{++}$  from intracellular stores further increasing  $\text{Ca}^{++}$  levels in the cytoplasm.

Two other mechanisms contribute to calcium overloading: exaggerated release of neurotransmitters like catecholamines and increased peroxidation of lipids. Monensin, lasalocid and salinomycin have been reported, respectively, to release catecholamines from adrenal chromaffin cells, transport catecholamines directly, or augment catecholamine plasma levels. The degradation products of catecholamines have been implicated in myocardial necrosis, through the formation of free radicals and calcium influx (Reichenback and Benditt, 1982). Since ionophores are lipophilic and have detergent properties, dose-related perturbations of plasma membranes can promote increased lipid peroxidation. It is known that lipid peroxidation promotes membrane damage, and consequently increases  $\text{Ca}^{++}$  influx.

Since they are not mutually exclusive, one or more of the above pathogenic mechanisms may increase calcium concentrations in the cell. The rise in intracellular calcium can be buffered by calcium pumps in the plasma membrane, mitochondria and sarcoplasmic reticulum. However, toxicity overwhelms this buffering mechanism and a vicious cycle ensues, resulting in calcium overloading. Elevated calcium levels then activate muscle proteases and phospholipases which initiate degradative processes (disassembly of myofilaments and membrane damage) in striated muscle and ultimately cell death (Van Vleet *et al.*, 1983c, 1991; Sandercock and Mitchell, 2004).



**FIGURE 97.4** Probable sequence of events induced by monensin in muscle cells. Adapted from Novilla and Folkerts (1986).

## Occurrence

Generally, the marketed ionophore products have been found to be safe and effective in the target species provided the approved dosage ranges. However, excessive ionophore feed concentrations have resulted in the ionophore toxic syndrome (Novilla, 1992). Toxic syndromes have occurred from the following: (1) feed mixing errors or ingestion of premix concentrates with unsafe amounts of ionophores; (2) extra label use, either accidental or intentional, have resulted in adverse reactions in horses, dogs, rabbits, adult poultry, ostriches, camels, deer, water buffaloes and humans; and (3) drug incompatibilities with other compounds such as tiamulin, chloramphenicol and macrolides such as triacetyloleandomycin have also resulted in toxicity (Novilla and Muller, 2003).

Feed mixing errors have caused the most toxicity problems in animals for which ionophore use has been approved. Reports of toxicity in the target species have



been reviewed (Potter *et al.*, 1984; Novilla and Folkerts, 1986; Dowling, 1992). Among the non-target species, horses appear to be the most susceptible and fish the most tolerant to high levels of ionophores. Except for a direct exposure to a monensin premix following a barn break-in, horse toxicity cases have resulted from feed contamination at the mill. Similarly, feed mill contamination of commercial dog food, cat food and concentrate ration for ostriches have resulted in toxicoses.

In humans, there are two publications of intentional exposure to monensin. According to Kouyoumdjian and associates (2001), a 17-year-old Brazilian male admitted ingesting monensin premix (Rumensin<sup>®</sup>, exact amount unknown), probably to develop muscle. Instead he fell ill, was hospitalized and died from acute rhabdomyolysis with renal failure. Although the amount of monensin ingested in this case was not estimated, in another case cited, two deaths among six people that consumed baked goods made with premix were attributed to monensin exposure of at least 10 times the optimum daily dose fed to cattle. In another report from Brazil, a 16-year-old farm worker who ingested approximately 500mg of monensin (5 grams of Rumensin<sup>®</sup> 100 premix) "to become stronger" developed an early and severe rhabdomyolysis followed by acute renal failure, heart failure and death (Caldeira *et al.*, 2001).

In target species, culprit feeds usually contained five times the maximum approved ionophore use level in the total mix ration provided to the affected animals, with two exceptions. Toxic episodes have occurred from amending cattle and sheep rations with poultry litter (30% or above) containing maduramicin residues and incompatibilities with drugs concurrently administered via feed or drinking water (Van Vleet, 1986; Dowling, 1992; Anadon and Reeve-Johnson, 1996; Novilla and Muller, 2003). Poultry litter contained levels of maduramicin which proved to be toxic to cattle and sheep. Various antibiotics, including tiamulin, chloramphenicol, macrolides and sulfa drugs, such as sulfachlorpyrazine, have been reported to potentiate ionophore toxicity. Among drug combinations that resulted in adverse effects in cattle, chickens (Frigg *et al.*, 1983), turkeys (Weisman *et al.*, 1983) and swine (Van Vleet, 1986), the most frequently reported drug interaction is with the pleuromotilin derivative, tiamulin. According to Meingassner *et al.* (1979), tiamulin interferes with the metabolic degradation of monensin in the liver, causing the ionophore to accumulate to toxic levels. The primary step in monensin metabolism is *o*-demethylation (Donoho, 1984) which is catalyzed by several P450 enzymes (Ceppa *et al.*, 1997; Nebbia *et al.*, 2001). Witkamp *et al.* (1996) considered the formation of tiamulin metabolite inhibitory (MI) complexes with P450 enzymes to be the basis of the toxic interaction, similar to that reported with macrolide antibiotics (see review

by Anadon and Reeve-Johnson, 1999). However, Szücs *et al.* (2004) reported no evidence of tiamulin MI complexes and that tiamulin directly inhibited CYP3A and two other enzymes, CYP1A2 and CYP2E1, involved in monensin-*o*-demethylation. Whether similar alterations in ionophore metabolism occur with other drugs are not clear but is an active area of research.

The incidence of ionophore toxicity in all species appears to be low (Novilla and Folkerts, 1986). This is based on periodic reviews of product complaints submitted to the U.S. FDA, as well as from a perusal of the literature. For instance, from 1976 to June 30, 1992, Elanco Animal Health received information about 168 complaints of cattle mortality involving 2045 head where Rumensin<sup>®</sup> was being fed and suspected as a cause of death. Other causes of death were ultimately found for all but 1532 head. Of the 1532 head, most deaths were believed to have been caused by mixing errors. Approximately one-half of the deaths occurred when there were mixing errors of greater than five times the intended dose. When compared to approximately 334 million head of beef cattle that received Rumensin<sup>®</sup> during this time period, the prevalence rate of toxicity would be less than 0.0005% (Novilla and Laudert, 2009). This is remarkable considering the widespread use of ionophore products. In the U.S., more than 90% of broiler chickens and about 75% of cattle marketed yearly have consumed ionophores at least part of their lifetime. More information and reports of adverse reactions are available on monensin than for the other ionophores because of monensin's long-standing and widespread use in food animals. However, all the other ionophores can produce a similar toxic syndrome following overdose, misuse and drug interaction.

Over the years, several acute toxicity (LD<sub>50</sub>) studies have been conducted in laboratory and domestic animals with monensin, lasalocid, salinomycin and narasin. Due to a change in FDA regulations, there are limited or no LD<sub>50</sub> data for the other marketed ionophores. Available data for most species are shown in Table 97.2.

The LD<sub>50</sub> of monensin varies from 214mg/kg in broiler chickens to 1.4mg/kg in horses, the most sensitive domestic animal species to ionophore toxicoses. In ionophore toxicoses, there is a progression of events from ingestion of a particular product. Dose and time factors influence the outcome. The greatest risk of intoxication is upon initial exposure, since animals will not consume highly contaminated feed. After an overdose, repeated daily intake of high levels of ionophores is not possible when anorexia occurs (Potter *et al.*, 1984; Matsuoaka *et al.*, 1996).

Laboratory and field studies have shown that there is a threshold dose for individual ionophores below which no adverse effects are observed. Safety studies have shown that consumption by horses of complete feed

TABLE 97.2 Acute oral toxicity of ionophores in various animals<sup>a</sup>

Animal species	Sex	Monensin <sup>b</sup>	Narasin <sup>c</sup>	Salinomycin <sup>d</sup>	Lasalocid <sup>e</sup>	Laidlomycin <sup>f</sup>
Chicken	C <sup>i</sup>	214 [100]	67	40	71.5	N.R. <sup>h</sup>
Turkey	C	253 [90]	N.D. <sup>g</sup>	N.R.	N.R.	N.R.
Cattle	MF <sup>j</sup>	26.4 [12.6]	N.D.	N.R.	< 10 > 50 [10]	N.R.
Horse	MF	1.38 ± 0.2 [0.675]	0.8	N.R.	21.5	N.R.
Swine	MF	16.7 ± 3.6 [4]	8.9	N.R.	N.R.	N.R.
Dog	M	[> 20]	[> 10]	N.R.	N.R.	N.R.
	F	[> 10]	(MF)			
Rabbit	MF	41.7 ± 3.6 [25.2]	[> 10.75]	N.R.	40	N.R.
Rat	M	40.1 ± 0.4	22	48.9	122	63
	F	28.6 ± 3.8	24	47.6	(MF)	(MF)
Mouse	M	70 ± 9	15.8 ± 2.6	57.4	N.R.	N.R.
	F	96 ± 12	16.7 ± 2.1	(MF)		
Sheep	MF	11.9 ± 1.2 [3]	N.D.	N.R.	N.R.	N.R.
Goat	MF	26.4 ± 4 [4]	N.D.	N.R.	N.R.	N.R.
Trout	MF	> 1000	N.D.	N.R.	N.R.	N.R.

<sup>a</sup>LD<sub>50</sub> – amount of drug required to kill 50% of the group of animals (LD<sub>0</sub> – no deaths) within 7 or 14 days after a single oral dose. No data available for maduramicin and semduramicin.

<sup>b</sup>Updated from Todd *et al.* (1984).

<sup>c</sup>From Novilla *et al.* (1994).

<sup>d</sup>Salinomycin sodium. Freedom of Information Summary. FDA NADA #D128686.

<sup>e</sup>From Galitzer *et al.* (1984); Lasalocid. Freedom of Information Summary. FDA NADA #96-298V.

<sup>f</sup>Laidlomycin propionate potassium. Freedom of Information Summary. FDA NADA #1410025.

<sup>g</sup>N.D. – Not Done.

<sup>h</sup>N.R. – No Record.

<sup>i</sup>C – combined LD<sub>50</sub> values.

<sup>j</sup>M – male, F – female, MF – males and females represented.

containing the maximum approved level of monensin for cattle is harmless. This is probably true for lasalocid and laidlomycin as well since there are no confirmed reports of ionophore toxicoses in horses used in cattle feedlots.

Results of acute toxicity studies with ionophores in domestic animals indicate that the horse is the most sensitive species; the chicken least sensitive; and cattle intermediate. From studies in ponies given toxic doses of monensin, Amend *et al.* (1981) found that the death pattern from single, high toxic doses by gavage varied from peracute death in less than 24 hours; acute death in 24 to 96 hours; subacute death in 4 to 14 days; and chronic death after 14 days. The latter is not a chronic toxicity by definition, but delayed death secondary to congestive heart failure (CHF), a recognized consequence of ionophore toxicoses. Death from CHF may occur in some animals that survive the acute toxic episode depending on the affected area in the heart and the cardiac reserve. In cattle, peracute death from monensin toxicity has not occurred. In fact, no deaths have been recorded earlier than 3 days even after high-level (LD<sub>50</sub>) toxic exposure (Potter *et al.*, 1984).

Blood-level studies suggest that horses, compared to cattle, are not able to clear monensin rapidly from the blood (Donoho, 1984). This was probably related to the oxidative efficiency of P450 demethylating enzymes in the liver. Working with liver microsomes from horses, pigs, broiler chicks, rats and cattle, Nebbia and associates (2001) found that horses had the lowest catalytic

TABLE 97.3 Effect of feeding monensin in complete feed to horses for 28 days

Monensin level	Effect
33 ppm (cattle use level)	Transient anorexia <sup>a</sup>
121 ppm (broiler use level)	May be toxic
330 ppm (10 × cattle use level)	Lethal

From Matsuoka *et al.* (1996).

<sup>a</sup>Occurred in week 2 and 3 of the study.

efficiency which may explain the greater susceptibility of horses to the ionophore-induced toxicosis, relative to the other species. However, not all levels of monensin are toxic to horses (Matsuoka *et al.*, 1996). In a sub-chronic feeding study in horses, all three horses provided a complete ration containing 330 ppm monensin (or 300 grams/ton, 10 times the maximum cleared level of monensin in complete feeds for feedlot cattle) and one out of three horses given 121 ppm monensin died during the 1-month feeding period. However, three horses provided 33 ppm monensin maintained typical levels of feed intake and suffered no ill effects attributable to treatment (see Table 97.3).

After evaluating the effects of monensin in either supplements or blocks in pasture horses, Matsuoka and co-workers (1996) made the following conclusions that may apply to other marketed ionophores as well: (1) horses on pasture may consume, at initial exposure, enough supplement containing high levels of monensin to be

toxic and/or lethal; (2) following an initial high-level monensin exposure, consumption of treated feed, supplement or block is negligible; and (3) horses can consume certain levels of monensin without suffering any ill effect indicative of toxicity.

## Clinical signs

Physical signs reported for ionophore toxicoses across several animal species are as follows: anorexia, diarrhea, depression, hypoactivity/reluctance to move, dyspnea, leg weakness, ataxia and recumbency. Most of these clinical signs occur in all animals studied but variations in their occurrence have been observed. For instance, diarrhea is commonly observed with monensin toxicity in cattle and poultry but has not been reported in either horses or dogs. The most consistent clinical sign in animals that have ingested toxic levels of ionophores is partial to complete anorexia (Novilla and Folkerts, 1986).

In chickens, anorexia is associated with diarrhea, drowsiness, extreme weakness and sternal recumbency with legs extended posteriorly. In target animal safety and toxicity studies, depression of growth and feed conversion as well as higher mortality occur in broilers fed five times the recommended level of monensin for 8 weeks (Novilla, 2004). Broiler breeders exposed to high levels of ionophores manifest severe drops in egg production, anorexia, depression, paralysis and death in sternal recumbency. Similar signs were reported in turkeys with toxicity induced by monensin, salinomycin and narasin (Salyi *et al.*, 1988). Mortality was variable but high death losses (up to 96.7%) were recorded with salinomycin (Halvorson *et al.*, 1982).

In cattle, clinical signs of monensin toxicosis include anorexia, diarrhea, ataxia, recumbency, depression and non-responsiveness to visual stimuli (Van Vleet *et al.*, 1983c; Potter *et al.*, 1984; Novilla and Muller, 2003). There is a definite correlation between onset of clinical signs and amount of monensin consumed. All intoxicated cattle manifest partial to complete anorexia. Severely intoxicated animals develop anorexia within 24 h. Animals exposed to lower levels may take up to 48 h to develop anorexia. Diarrhea usually is evident in 24 to 48 h in animals receiving high doses of monensin. Exposure to low doses may result in a delay of up to 5 days in the onset of diarrhea. Onset of depression follows a similar pattern, being observed by day 3 in animals receiving excessive monensin overdoses while being observed after 1 week in animals receiving less than excessive overdoses. Rapid breathing and ataxia may be present only in animals that have consumed extremely high levels. In cattle, death occurs without any manifestations of struggle.

Galitzer and coworkers (1982) found that lasalocid-intoxicated cattle manifested early signs of forced watery

diarrhea, muscle tremors and greater cardiac and respiratory rates followed by or concurrent with anorexia. Delayed signs of cardiac insufficiency were evident 7 to 10 days after administration of the toxic dose. The temporal occurrence of clinical signs and mortality from ionophore toxicity was clearly documented by Potter *et al.* (1984) in a multiple bolus dosing trial with monensin in cattle (see Table 97.4).

The onset and severity of toxic effects induced by monensin were dose related. In the study, anorexia was the most consistent clinical sign of toxicity and no deaths occurred earlier than 4 days after exposure to high toxic doses of monensin. However, multiple bolus dosing does not simulate actual field conditions since repeated exposure to toxic levels is not possible when anorexia occurs. Further, the onset and severity of toxicity from bolus dosing is different than that via feed (Buck *et al.*, 1976).

Sheep and goats have clinical signs similar to cattle (anorexia, diarrhea, ataxia) but affected lambs frequently exhibit labored breathing, frothing at the mouth and kicking at the abdomen (Agaoglu *et al.*, 2002; Novilla and Muller, 2003). Dogs with ionophore toxicoses become weak and develop a rapid onset of paresis progressing to flaccid paralysis of the anterior and posterior limbs, tongue laxity and dyspnea (Safran *et al.*, 1993; Condon and McKenzie, 2002; Novilla and Muller, 2003; Segev *et al.*, 2004). In pigs, anorexia, diarrhea, lethargy, dyspnea, ataxia, knuckling at the fetlock and myoglobinuria were present prior to lateral recumbency and death from ionophore toxicoses (Van Vleet *et al.*, 1983a,b; Plumlee *et al.*, 1995).

As in cattle and other species, anorexia is the first and most consistent clinical sign finding in the horse with ionophore toxicoses (see Table 97.5).

Diarrhea has not been observed with either monensin or lasalocid but was reported with salinomycin and narasin (Whitlock *et al.*, 1978; Novilla and Folkerts, 1986; Matsuoka *et al.*, 1996; Novilla and Muller, 2003). Profuse sweating was also observed with monensin, salinomycin, narasin and laidlomycin but not with lasalocid toxicity. Horses with leg weakness and ataxia generally progress to recumbency. Once recumbent, horses frequently attempt to rise with thrashing of the limbs until death supervenes. This is in contrast to cattle that die without signs of struggling.

## Post-mortem findings

Necropsy findings in animals with ionophore toxicoses include hemorrhages and pale areas in the heart, pale areas in some limb muscles, pulmonary edema, hydrothorax, ascites and inflammation of the stomach and intestines (Van Vleet *et al.*, 1983a,b,c; Galitzer *et al.*, 1986a,b; Novilla and Muller, 2003). Animals that die

TABLE 97.4 Clinical signs in beef cattle gavaged with multiple doses (7 days) of monensin

Monensin mg/kg/day	Day first observed					
	Anorexia	Diarrhea	Depression	Dyspnea	Ataxia	Death
0	— <sup>a</sup>	—	—	—	—	—
1.5	2	5	7	—	—	—
2.3	1	4	6	—	—	—
3.8	1	3	4	—	—	—
7.6	1	2	4	5	5	5
15.5	1	2	3	5	4	4

<sup>a</sup>Not observed.

TABLE 97.5 Clinical signs of ionophore toxicoses in the horse

Sign	Monensin	Lasalocid	Salinomycin	Narasin	Laidlomycin
Anorexia	X <sup>a</sup>	X	X	X	X
Diarrhea	— <sup>b</sup>	—	X	X	—
Depression	X	X	—	X	X
Dyspnea	X	—	X	X	X
Ataxia	X	X	X	X	X
Colic <sup>c</sup>	X	—	X	X	—
Leg weakness	X	X	X	X	X
Muscle tremors	X	—	—	X	X
Sweating	X	—	X	X	X
Recumbent	X	X	X	X	X

<sup>a</sup>Observed.<sup>b</sup>Not observed.<sup>c</sup>Pain manifested by uneasiness, abnormal (wide) leg stance, pawing.

soon after exposure often will have no lesions, since they had not had time to develop or they may be masked by post-mortem changes. Those with lethal doses surviving longer than a week may have hydropericardium, pulmonary edema, hydrothorax, ascites, reddish-mottled liver and subcutaneous edema of the abdomen and limbs. (See Figures 97.5 and 97.6.)

At necropsy, cutting the ventricular and thigh muscles transversely often shows the diffuse pallor of myonecrosis induced by toxic levels of ionophores. Skeletal muscle lesions may be quite severe in sheep, pigs and dogs, moderate in cattle and minimal to slight in horses. For this reason, myoglobinuria, evidenced by red urine, has been observed in pigs, sheep and dogs but not in cattle or horses.

## Histopathologic findings

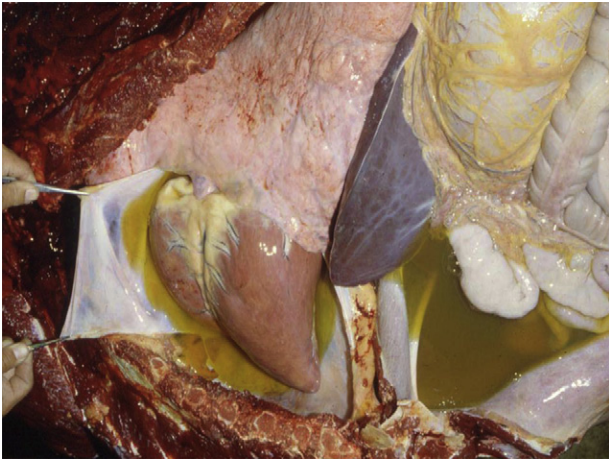
Target organs damaged by toxic doses of monensin and other ionophores were identified to include the heart and skeletal muscles in all species studied (Todd *et al.*, 1984; Novilla and Folkerts, 1986; Van Vleet *et al.*, 1991; Dowling, 1992; Novilla and Muller, 2003). In addition, neurotoxic effects have been reported for lasalocid (Shlosberg *et al.*, 1985; Safran *et al.*, 1993), narasin (Novilla *et al.*, 1994) and salinomycin (Van der

Linde-Sipman, 1999). The development of muscle lesions varies among species. The heart is primarily affected in horses, skeletal muscle in pigs and dogs, and there is about equal tissue predilection in rats, chickens and cattle. Morphologic effects include degeneration, necrosis and repair of cardiac and skeletal muscle fibers with a variable inflammatory component and secondary lesions of congestive heart failure. Neuropathic changes occurred in peripheral nerves and the spinal cord. Focal swelling, fragmentation, loss of axons and formation of digestion chambers filled with macrophages were observed in both sensory and motor nerves, and there was vacuolation with swelling, degeneration and fragmentation of myelin sheaths and axons in the spinal cord.

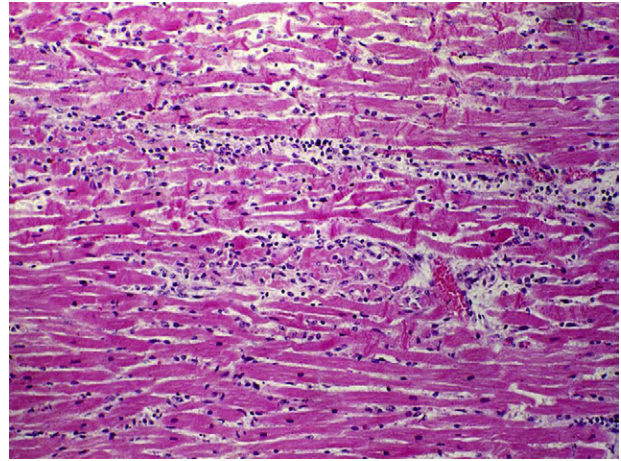
The most important change is a toxic myopathy characterized by focal areas of degeneration, necrosis and repair in cardiac and skeletal muscles with a variable inflammatory component (Novilla and Folkerts, 1986; Van Vleet *et al.*, 1991). Muscle lesions are similar to those described for compound A204, the first polyether antibiotic tested at Lilly Research Laboratories (Todd *et al.*, 1984). Doses and time factors influence the severity and distribution of lesions in ionophore toxicoses. (See Figures 97.7 and 97.8.)

Generally, no significant lesions are seen by light microscopy in animals that die immediately, and animals





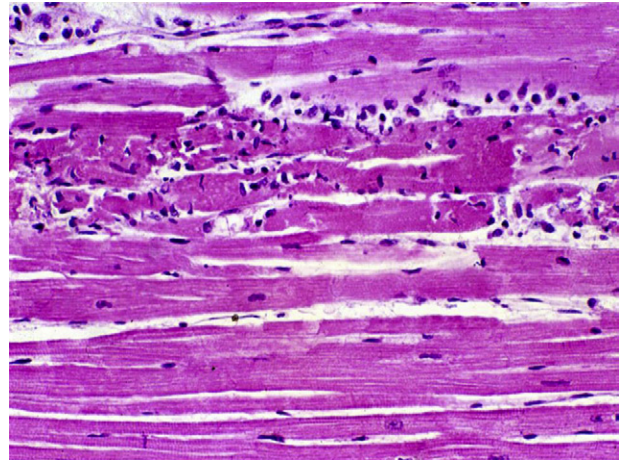
**FIGURE 97.5** Thoracic and abdominal viscera from a mare that died 7 days following a single gavage dose of 1.65 mg monensin/kg body weight. Note the fluid accumulation in the body cavities and the pale areas and epicardial hemorrhages on the heart.



**FIGURE 97.7** Early necrotic focus in the left ventricle of a gelding that died 20 hours following gavage with 2.5 mg monensin/kg body weight. Necrotic muscle fibers have sparse infiltration of neutrophils and lymphocytes. H&E. Original magnification  $\times 64$ .



**FIGURE 97.6** Diffuse pallor in cross-sections of the heart from the same mare described in [Figure 97.5](#) legend.



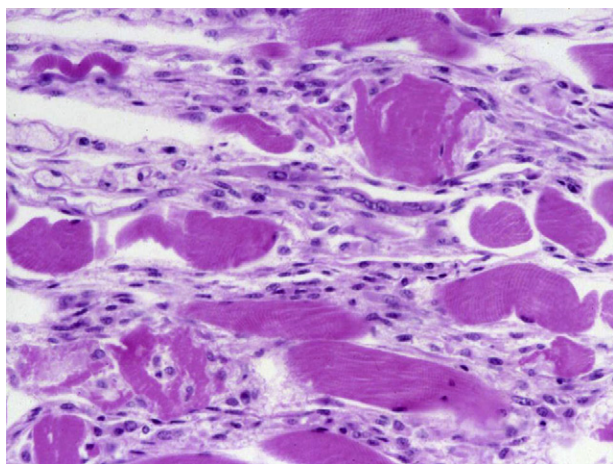
**FIGURE 97.8** Larger foci of myofiber necrosis with contraction bands in the interventricular septum from a horse euthanized 72 hours following a single oral dose of 1.65 mg monensin/kg body weight given by gavage. More cellular infiltrates with lymphocytes, macrophages and few eosinophils are present. H&E. Original magnification  $\times 10$ .

that die after an acute course may have only a few scattered degenerated fibers in the heart and highly active muscles, such as the diaphragm. Lesions are most pronounced within 7 to 14 days following ingestion of a toxic dose and are accompanied by profound attempts at repair. Skeletal muscle fibers regenerate rapidly, and

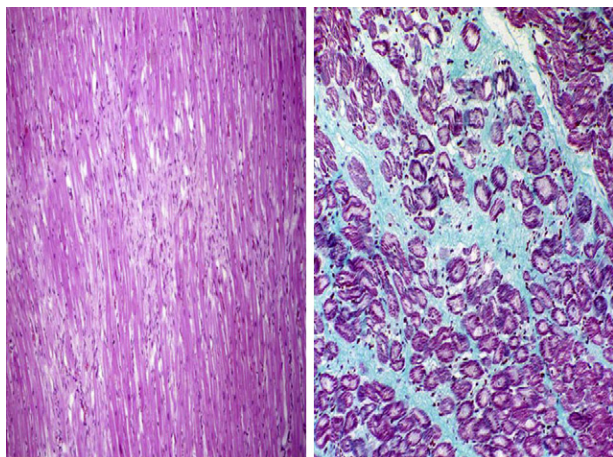
lesions are completely healed in about a month. Heart muscle fibers do not regenerate, but repair takes place by replacement fibrosis. Secondary lesions of congestive heart failure, including heart dilatation and hydropericardium, lung edema, liver necrosis and fluid accumulation in the thoracic and abdominal cavities, may be present, depending upon the severity of heart damage and the potential cardiac reserve. (See [Figures 97.9](#) and [97.10](#).)

Observations to date indicate that in horses the heart suffers the greatest damage from monensin toxicoses with little or no involvement of skeletal muscles. This finding is in contrast to dogs and pigs in which





**FIGURE 97.9** Skeletal muscle from a steer that died 6 days after a gavage dose of 39.8mg monensin/kg body weight. Notice the fragmented and regenerating fibers. H&E. Original magnification  $\times 80$ .



**FIGURE 97.10** Hearts from animals that survived acute ionophore toxicity. (Left panel) Section of left ventricle with focal interstitial fibrosis from another steer, cohort of that described in Figure 97.9 legend, euthanized 28 days after a gavage dose of 39.8mg monensin/kg body weight. H&E. Original magnification  $\times 20$ . (Right panel) Interstitial fibrosis in the left ventricle from a gelding that survived a gavage dose of 2mg monensin/kg body weight for 4 months. Masson's trichrome. Original magnification  $\times 32$ .

the lesions are most pronounced in skeletal muscles. Chickens, cattle and rodents have about equal predilection for cardiac and skeletal muscle lesions.

### Other laboratory findings

Mitochondrial swelling, myelin figures and lipid vesicles were observed ultrastructurally in cardiac and skeletal muscles of ponies given monensin at 4mg/kg and observed for 79h (Mollenhauer *et al.*, 1981). Early

**TABLE 97.6** Some biomarkers of ionophore toxicity

Elevated	Decreased
AST	Calcium
CK	Potassium
LDA	No change
Alkaline phosphatase	Sodium
BUN	
Bilirubin	

degenerative changes of sarcoplasmic vacuolation from swollen mitochondria and accumulation of lipid were described in cattle and pigs with monensin toxicosis (Van Vleet *et al.*, 1991). Severely injured mitochondria had marked swelling, disrupted cristae and dense matrical granules. Scattered myelin figures were present in muscle cells with numerous disrupted mitochondria. Subsequently necrosis occurred in degenerated myocytes and was characterized by dense fibers with intact sarcomeres or disrupted fibers with hypercontraction bands. Necrosis was followed rapidly by extensive macrophage infiltration with lysis of disrupted organelles and contractile material. Similar ultrastructural findings were observed in hearts and skeletal muscles of sheep given monensin and in skeletal muscles of broilers simultaneously given triacetyloleandomycin in the drinking water and monensin in the feed (Dowling, 1992). Regeneration as evidenced by myoblast proliferation was observed as early as 4 days post-treatment in pigs and sheep.

Hematologic parameters are not significantly affected by ionophore toxicosis (Van Vleet *et al.*, 1983a,b,c; Condon and McKenzie, 2002; Segev *et al.*, 2004). Elevation of serum enzymes, notably creatine kinase, lactate dehydrogenase and aspartate transaminase, may indicate damage to cardiac and skeletal muscles (Amend *et al.*, 1981; Van Vleet *et al.*, 1983b,c; Galitzer *et al.*, 1986b; Wilson, 1990). Alkaline phosphatase, inorganic phosphorus and total bilirubin levels are also higher, while serum levels of calcium and potassium are lower. (See Table 97.6.)

The progressive hypokalemia and attendant cardiac conduction disturbances demonstrated in ponies were considered the life threatening events in early acute monensin toxicosis (Amend *et al.*, 1981).

### Diagnosis and differential diagnosis

Since all ionophores in the market place are likely to produce a similar toxic syndrome in overdosage and misuse situations, six important criteria must be considered before a diagnosis of toxicity is given (Novilla and Muller, 2003). These include (1) history of feed-related problem, usually affecting a group of animals; (2) ionophore laboratory assays; (3) clinical signs manifested during the toxicity episode; (4) gross post-mortem

lesions; (5) microscopic pathology; and (6) exclusion of nutritional, infectious and other toxic factors.

History assumes great significance when the problem is connected to the introduction of newly formulated feed or supplement to the herd or flock. Since clinical signs and lesions are not pathognomonic, feed analysis for the amount and type of ionophore in the ration is necessary for diagnosis. With the availability of seven ionophores in the market place, the use of an efficient and highly selective laboratory assay is indicated. Newer methods have been developed to determine one or more ionophores in feeds and tissues (Bertini *et al.*, 2003; Ebel *et al.*, 2004). Several samples of the feed should be submitted for analysis, including residual material from the feeder, trough, auger, or feed bin. Feed assays may prove exposure to a particular product but significantly higher than the recommended levels must be found for a confirmatory diagnosis. Otherwise, concurrent use of an incompatible drug must be documented. Absent proof of a gross feed mixing error, a wide list of differential diagnoses need to be excluded in order to return a presumptive diagnosis.

Initially, ionophore toxicoses may be suspected when there is a history of a feed-related problem in a group of animals; clinical signs of anorexia, diarrhea, labored breathing, depression, locomotory disorder, recumbency and death; lesions affecting heart and skeletal muscles; or congestive heart failure. The clinical signs and lesions induced by toxic levels of ionophores are not pathognomonic. However, recent introduction of newly formulated feed or supplement to a flock or herd in which signs and lesions are present may cause one to suspect that acute intoxication has occurred. Dose and time factors influence the severity and distribution of lesions. Animals that die soon after exposure may not have muscle lesions discernible by light microscopy. Lesions are likely to be found in animals that survived longer than a week. The most active skeletal muscles may be involved when the heart is not affected or is only slightly affected. Since changes can be missed because of their focal distribution, more intense tissue sampling to include one section each of the atria, ventricles and interventricular septum of the heart, the diaphragm and muscles of the abdomen and thigh is desirable. Some animals with substantive heart damage from very high levels of monensin and other ionophores may later develop congestive heart failure.

Although a presumptive diagnosis of ionophore toxicosis can be made based on history, clinical signs, lesions and considerations of differential diagnosis, specific assays are needed for confirmatory diagnosis. With seven ionophores currently in use, the need for confirmatory laboratory assays cannot be overemphasized. In monensin toxicosis, values greater than five times the recommended use level in the feed provided affected animals are usually confirmatory. Assays on stomach contents from per acute and acute cases of toxicity can prove

exposure but values obtained have been low. Only minimal residues of monensin have been detected in target tissues of cattle and chickens given monensin (Donoho, 1984; Atef *et al.*, 1993). Further, blood levels of monensin are low or undetectable even in intoxicated animals and accumulation in target tissues does not occur.

Ionophore toxicosis may be confused with acute infectious diseases, deficiencies and other intoxications (Van Vleet *et al.*, 1983a,b,c; Novilla and Folkerts, 1986; Dowling, 1992; Novilla and Muller, 2003). In the differential diagnosis of monensin toxicosis, myopathic conditions should be considered first. In cattle, these would include (1) ionophore toxicoses, (2) vitamin E and selenium deficiencies, (3) poisonous plant ingestion, e.g., senna, coyotillo, white snakeroot, vetch, and (4) the common, yet puzzling, sudden death syndrome with myocardial necrosis. Ionophore toxicosis usually involves an accompanying history of feed supplementation or feed change and usually involves many animals. Clinical signs are anorexia, diarrhea, lethargy, ataxia and the suggestion of damage to striated muscles. Vitamin E and selenium deficiency occurs sporadically and produces prominent degeneration and necrosis with calcification of cardiac and skeletal muscles. Plant poisonings are usually localized to areas where the toxic plants are indigenous. For instance, coffee senna (*Cassia occidentalis*) poisoning occurs in the southeastern United States. It may cause anorexia, diarrhea and the production of dark urine, but generally causes more pronounced lesions in skeletal muscles than in the heart. The coyotillo plant (*Karwinskia huntholtiana*) in southwest Texas and Mexico produces limberleg in sheep and goats characterized by progressive weakness of the legs, muscular incoordination, recumbency, respiratory distress and death. Lesions are observed both in cardiac and skeletal muscles as well as peripheral nerves and the liver. White snakeroot (*Eupatorium rugosum*), a plant indigenous to much of eastern Canada and the United States, causes "trembles" in goats, sheep, cattle, horses and swine. Cardiac and skeletal muscle lesions may be present in animals that ingested this plant. However, with trembles there is constipation, blood in feces, an odor of acetone in the breath and severe fatty degeneration in the liver and kidney that are not seen in ionophore toxicities. Hairy vetch (*Vicia villosa*) also produces myocardial necrosis but, unlike monensin toxicosis, it produces dermatitis, conjunctivitis and abortion as well as lesions in the kidneys, adrenal glands, lymph nodes and thyroid gland. The syndrome of sudden death with myocardial necrosis in cattle, especially calves, is common but sporadic in occurrence and is associated with lesions in cardiac but not skeletal muscle (Bradley *et al.*, 1981). Hence, clinical history and detailed pathologic studies will help distinguish among ionophore toxicosis, acute infectious diseases, deficiencies and other intoxications.

From a clinical standpoint, respiratory diseases, particularly infectious bovine rhinotracheitis and the shipping fever complex, are initially considered in the differential diagnosis for cattle because of the respiratory difficulties that occur with ionophore toxicosis. At necropsy, however, pneumonic lesions are consistent with these diseases. Animals with acute bovine pulmonary edema and emphysema or fog fever, nitrogen dioxide intoxication, or rape, turnip, or kale poisoning also exhibit respiratory difficulties; but, in these conditions, gross lesions will include severe interstitial and interlobular emphysema or pneumonia or both rather than edema. The incoordination, stiff wobbly gait and loss of visual reflexes may lead one to suspect polioencephalomalacia and thromboembolic meningoencephalitis but, in these conditions, histologic lesions present in the brain are confirmatory. Unequivocal central nervous system lesions have not been found in cases of monensin toxicity in any species. However, lesions in the spinal cord and peripheral nerves occurred with lasalocid toxicosis in chickens (Shlosberg *et al.*, 1985). Peripheral neuropathic changes have also been reported in cats exposed to salinomycin-contaminated feed (Van der Linde-Sipman *et al.*, 1999) and in dogs given narasin (Novilla *et al.*, 1994). Salt poisoning will cause nervous signs, paralysis and diarrhea, but knowledge that insufficient amounts of water were available to the animals will also point to this problem. Eosinophilic meningoencephalitis is pathognomonic for salt poisoning in pigs, but this lesion does not occur in other species. Laboratory procedures used to confirm a diagnosis include assays of serum, cerebrospinal fluid and brain tissue for sodium concentrations (Buck *et al.*, 1976). In cattle, urea toxicosis must be considered when sudden collapse, bloat, violent convulsions, terminal tetanic spasms and high death losses occur within 10 min to 4 h from exposure to newly formulated feed. Deaths occurring earlier than 72 h have not been reported in cattle gavaged with high doses of monensin (Potter *et al.*, 1984).

In poultry, differential diagnoses should include nutritional (focal) myopathy, coffee senna toxicity, botulism, sodium chloride (salt) toxicity, mycotoxicosis by cyclopiazonic acid and other myopathic mycotoxins, round heart disease and, in the turkey, the knockdown (downer) syndrome. Although no striated muscle lesions are produced in botulism and sodium chloride toxicity, clinical signs of limber neck and lesions of "water belly" may be confused with ionophore toxicoses. On the other hand, birds affected with salt poisoning may have enlarged hearts or have enlarged pale kidneys from urate nephrosis. Monensin *per se* does not produce "barebacks" in broiler chickens, and downers among replacement birds may be suffering from viral arthritis. Therefore, this common reovirus infection must be excluded as a cause of the problem in chicken flocks. In commercial turkeys reared in confinement, focal myopathy has been attributed to

deficiencies in vitamin E or selenium associated with rapid growth (Wilson *et al.*, 1990). Nutritional myopathy may mimic skeletal muscle lesions induced by ionophore toxicoses; but unlike those of nutritional myopathy, ionophore-induced lesions are monophasic and polyfocal with little or no mineralization. Muscle lesions also occur in deep pectoral myopathy, coffee senna toxicity and toxicoses from the mold toxins, cyclopiazonic acid and moniliformin. Their clinical presentation and presence of other lesions will help distinguish these conditions.

Round heart disease, also known as round heart-edema-ascites syndrome or spontaneous cardiomyopathy, occurs sporadically in turkeys and other fowl (Czarnecki, 1984). Clinical signs indicate the presence of heart failure but the exact cause has not been determined. A genetic predisposition has been suggested because it is more common in certain lines of turkeys, particularly those having a rapid rate of early muscle development. Some of the predisposing factors include low levels of oxygen and high levels of carbon dioxide in the incubator, poor brooder house ventilation and overcrowding during transport. Exposure to these factors may occur days or weeks prior to onset of illness. In contrast, monensin toxicity occurs as a sudden outbreak in a flock of birds accidentally provided toxic levels due to a feed mixing error. Among birds that survive the acute toxic episode, a few, depending upon the extent and location of heart damage and the potential cardiac reserve, may subsequently develop dilatation of the heart and secondary signs of congestive heart failure.

Another condition that needs to be distinguished from monensin toxicity is the turkey knockdown syndrome. Turkey knockdown has been defined as any condition affecting the neuromuscular system to the extent that a bird is unable to stand or walk (Wages, 1993). A necrotizing myopathy, particularly of the rear limbs, has been reported in turkeys with the knockdown syndrome (Cardona *et al.*, 1993). Unfortunately, striated muscles have limited responses to injury (Van Vleet *et al.*, 1991) and lesions induced by any ionophore, including monensin, are not pathognomonic (Novilla, 1992). Although monensin feed inclusion rates in knockdown cases are within or slightly above the maximum recommended level of 99 ppm (Wages, 1993; Cardona *et al.*, 1993) monensin has been implicated as a causative factor because the clinical and pathologic findings are similar to those induced by monensin toxicity. However, unlike monensin toxicity, the turkey knockdown syndrome is characterized by: (1) low incidence and fatality rates; (2) acute onset of paresis/paralysis; (3) no anorexia, birds will eat if able to reach feeders; (4) no diarrhea; (5) affected birds are alert; (6) unable to vocalize; (7) presence of widespread skeletal muscle lesions that are subacute to chronic in character, generally no heart lesions; and (8) rapid recovery following change of feed. These



observations were confirmed in an epidemiological, case controlled study of knockdown cases in the Shenandoah Valley of Virginia that evaluated various factors, including management, environment, health, feed, water, pathology and clinical pathology analytes (Evans *et al.*, 2000). Serum vitamin E levels were significantly lower in affected birds (Meldrum *et al.*, 2000). Feed and/or water intake alterations were highly correlated with the incidence of turkey knockdown and modification of practices affecting feed/water intake reduced the incidence of knockdown in one farm from 6 in 1999 to 0 in 2000.

In pigs, vitamin E and selenium deficiency, gossypol poisoning and porcine stress syndrome (PSS) should be considered in the differential diagnosis since skeletal or cardiac lesions may be found in these conditions (Van Vleet *et al.*, 1983b, 1986). In monensin toxicosis, the striated muscle lesions appear more frequently and are more severe in skeletal muscle than in cardiac muscle. Myoglobinuria may also be present. Widespread cardiac and skeletal muscle lesions, vascular damage, dietetic hepatitis and gastric ulceration may be found in vitamin E and selenium deficiency. With PSS, cardiac and skeletal muscle lesions may or may not be observed following a history of stress.

For horses, the exertional myopathies, such as equine rhabdomyolysis (Monday morning disease) and hyperkalemic periodic paralysis, plant poisoning from coffee senna and white snakeroot, should be excluded along with blister beetle intoxication, colic and laminitis (Whitlock *et al.*, 1978; Amend *et al.*, 1981; Novilla and Folkerts, 1986). Complete herd history, clinical examination, successful supportive treatment and necropsy may help differentiate these conditions from ionophore toxicoses.

## TREATMENT

When ionophore toxicity is suspected, a feed change to a non-ionophore medicated ration must be made immediately and the affected group(s) of animals maintained on this ration until all diagnostic procedures are completed. In cattle presumably diagnosed with monensin toxicity, it is advisable to replace the suspected culprit feed with feed known to contain the approved concentration of monensin. This allows the rumen microbes of unaffected cattle to remain adapted to monensin and will promote overall herd performance (Novilla and Laudert, 2009). At the present time, there is no known antidote or treatment for ionophore toxicity. Although previous antidotal trials failed, the search continues (Mitema *et al.*, 1988). Nonetheless, it may be difficult to find a drug that can block up to four pathogenic mechanisms, acting singly or in concert, that are involved in the toxicoses.

Therapeutic interventions have been largely supportive. For horses, Amend *et al.* (1981) recommended supportive treatment with mineral oil, activated charcoal and aggressive fluid and electrolyte replacement with potassium and phosphorus supplementation, including means to reduce cardiac work. However, supportive therapy may not be practical on a herd or flock basis.

Van Vleet and coworkers (1983a, 1986, 1987) obtained protection against the development of *clinical* monensin toxicity in pigs treated with selenium and vitamin E (selenium as selenite at 0.25mg/kg and vitamin E as  $\alpha$ -tocopherol acetate at 17IU/kg) prior to a single oral dose of 50mg monensin/kg ( $LD_{50}$   $16.7 \pm 3.57$  mg/kg). A similar regimen in cattle administered toxic doses of monensin provided only partial protection but clinical signs in pigs with tiamulin-induced salinomycin toxicity were ameliorated following administration of vitamin E. The protection against the toxicoses was theorized to be produced by stabilization of cellular membranes since selenium and vitamin E are known to prevent and control peroxidation-mediated cellular injury (Tappel, 1981; Van Vleet, 1986). Similarly, the antioxidant property of zinc prevented adverse effects of salinomycin in chickens (Kamashi *et al.*, 2004). Monensin, like all polyether carboxylic ionophores, is lipophilic and may produce dose-dependent membrane perturbations and increased lipid peroxidation, which could lead to degradative processes. Further studies are needed to determine whether antioxidants, such as vitamin E and selenium, or zinc administration may have important roles in the prevention and treatment of ionophore toxicoses.

Until proper and effective therapy is available, prevention of ionophore toxicoses by (1) proper use from the implementation of good feed manufacturing and feeding practices at the feed mill and farm level; (2) avoidance of overdosing of feeds for approved species, which may carry over to feeds for non-target species; and (3) adherence to species restrictions will help prevent the adverse effects associated with this class of compounds.

## CONCLUSIONS

Seven ionophores – monensin, lasalocid, salinomycin, narasin, maduramicin, laidlomycin and semduramicin – are marketed globally for use as anticoccidial drugs for poultry and/or growth promotants in ruminants. Off-label usage of ionophore products is known to occur since other uses continue to be investigated and applied in many countries. It is likely that basic and applied research on these versatile compounds could lead in the future to product line extensions to other target species and potential development of novel therapeutics for unmet needs in veterinary and human medicine.

Generally, these feed additives have been found to be safe and effective in target animal species, but toxic syndromes have resulted from overdosage, misuse and drug interaction. Among the domestic species, horses are the most sensitive to ionophore toxicoses, poultry the least sensitive, and cattle intermediate. However, even for the horse, there is a threshold level of exposure below which no adverse effects are observed. Consumption of complete feed containing the maximum approved use levels of monensin, lasalocid, or laidlomycin is harmless. Dose and time factors influence the severity and outcome of the toxic exposure. Results of controlled studies and confirmed field reports of toxicity indicate that the greatest risk of intoxication is upon initial exposure to ionophore-containing feed or supplement. Following sublethal exposure, consumption of culprit feed or supplement is negligible because of anorexia. Animals that die acutely after high levels of exposure often will have few or no lesions. Those that die later have profound striated (cardiac and/or skeletal) muscle lesions and changes secondary to congestive heart failure in some animals that survive the acute toxic episode.

Confirmatory diagnosis requires efficient laboratory assays to determine the identity and amounts of the ionophore involved and a thorough consideration of differential diagnosis. These cannot be overemphasized. There is no known antidote or specific treatment for ionophore toxicoses and treatment is largely supportive. Judicious use, avoidance of overdosing and adherence to species recommendation will enhance livestock production and help prevent the occurrence of adverse effects associated with this class of compounds.

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## Nonprotein nitrogen (urea) and hyperammonemia

*Rhian Cope*

### INTRODUCTION AND BACKGROUND

Nonprotein nitrogen (NPN) intoxication is not a common occurrence in ruminants. It has long been recognized that ruminal microbes of cattle and sheep can utilize NPN to synthesize proteins that can replace a portion of their total dietary protein requirements. Given that feeding NPN sources are typically cheaper than feeding expensive true protein, the practice of adding NPN to ruminants' diets is relatively commonplace. Feed grade urea ( $\text{CO}[\text{NH}_2]_2$ ) is the cheapest, most effective and most available nitrogen source. Other NPN compounds, which may be less readily available and more expensive, include ammonium acetate ( $\text{CH}_3\text{CO}_2\text{NH}_4$ ), ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), ammonium carbamate ( $\text{NH}_2\text{CO}_2\text{NH}_4$ ), ammonium lactate ( $\text{CH}_3\text{CHOHCO}_2\text{NH}_4$ ), ammonium sulfate ( $[\text{NH}_4]_2\text{SO}_4$ ), monoammonium phosphate ( $\text{NH}_4\text{H}_2\text{PO}_4$ ), biuret ( $\text{NH}_2\text{CONHCONH}_2$ ), dicyanodiamide ( $\text{NH}_2\text{C}[\text{NH}]\text{NHCN}$ ) and diammonium phosphate ( $[\text{NH}_4]_2\text{HPO}_4$ ). All these NPN sources consist of varying nitrogen content and protein equivalent; so addition of these products into the diet requires stringent nutritional dietary examination. Many of these supplements are solids and can be directly mixed into the mineral or grain component of the diet. Some, such as urea, can be added to molasses-based liquid products or into solid mineral blocks. Ammoniated beet pulp, citrus pulp, straw, silage, molasses and rice hulls have also been used to supplement the protein content of a ruminant's diet. Ruminants can also be poisoned by gaining access to some fertilizers that can contain over 40% urea.

### TOXICITY

All mammalian species are potentially susceptible to NPN poisoning via the consumption of ammonium-containing feeds, if the dose is high enough. When urea is added to the ruminant's diet, it is rapidly broken down by bacterial urease in the rumen to ammonia. The ammonia is then utilized by the rumen bacteria, along with soluble carbohydrates, to synthesize amino acids and proteins (hence the name "nonprotein nitrogen"). Most of the excess ammonia produced is protonated to the ammonium ( $\text{NH}_4$ ) ion and is trapped in the rumen. If this excess ammonia production continues, the rumen pH increases. When ammonia concentrations become too high too quickly, significant amounts get absorbed systemically. In the bloodstream, ammonia travels via the portal circulation to the liver where it is incorporated into the urea cycle and urea is then excreted in the urine via the kidneys or secreted into the saliva. Toxicities occur when this entire system becomes overwhelmed resulting in a clinically significant hyperammonemia. Excess ammonia has been shown to inhibit the citric acid cycle ([Hatch, 1977](#)), to cause a lactic acidosis and to interfere with cerebral energy metabolism and the sodium-potassium ATPase pump. The systemic metabolic acidosis is thought to be related to a hyperkalemia, and it is this abnormality that can ultimately lead to cardiac arrest.

Factors that potentially can predispose ruminants to poisonings with these compounds include low energy diets, high rumen pH (e.g., rumen impaction), elevations in body

temperature (enhances urease activity), dehydration, stress, concurrent disease, alterations in rumen microflora and hepatic insufficiency. The lack of a readily available supply of soluble carbohydrates markedly affects the rumen bacteria's ability to utilize the ammonia. It is generally recommended to slowly introduce increasing levels of NPN in the diet over a period of several days to allow the rumen bacteria to adapt to the ammonia source. Most poisonings in ruminants occur as a result of inadequate implementation of an adaptation period, improper mixing of the supplement into the diet and unlimited/unrestricted access to palatable liquid NPN supplements. NPN poisoning in monogastrics is not common.

Toxic and lethal doses are difficult to establish in ruminants due to all the predisposing factors that can enhance or decrease their sensitivity to these compounds. The oral lethal dose for urea in horses is 4.0g/kg body weight and the oral lethal dose for ammonia salts is 1.5g/kg body weight (Hintz *et al.*, 1970). A reported oral toxic dose for urea in nonacclimated cattle is 0.44g/kg body weight (Word *et al.*, 1960), whereas a lethal dose is in the 1.0–1.5g/kg body weight range (Osweiler *et al.*, 1985). Urea is recommended in ruminant rations at a rate of approximately 3% of the grain ration or 1% of the total ration. Adapted cattle can tolerate much higher levels in their diets. Biuret is considered one of the safest of NPN compounds, and a reported lethal dose in cattle is 8.0g/kg body weight (Haliburton and Morgan, 1989). A single dose of 116g of urea caused clinical signs of toxicity in cattle, whereas a dose of 57g did not (Dinning *et al.*, 1948). Davis and Roberts (1959) reported that 0.3g/kg body weight urea was toxic to unacclimated cattle.

## CLINICAL SIGNS

The onset of clinical signs following introduction of a toxic NPN source into an animal's diet can be extremely quick; frequently within 30 min. A delay in the onset of clinical signs can be due to a wide variety of factors, including dose, method of introduction and degree of adaptation. In general, most cases of NPN poisoning in ruminants involve some history of some type of "recent" feed change. Commonly reported clinical signs include uneasiness and ataxia, muscle tremors, excessive salivation, weakness, labored breathing, abdominal pain and bloat. The progression of clinical signs is generally quite rapid and recumbency, convulsions and death typically ensue within 4 h. Hyperthermia, marked jugular pulse, cardiac arrhythmias, vomiting/regurgitation and cyanosis have also been reported (Haliburton and Morgan, 1989). Rarely do animals survive once clinical signs start without some type of rapid treatment intervention.

## DIAGNOSTIC CRITERIA

The diagnosis of NPN intoxication rests mainly on a compatible history of exposure to an NPN source, compatible clinical signs with rapid progression to death and clinical diagnostic laboratory data. Rarely do you find significant post-mortem changes. Common findings at necropsy include generalized congestion of visceral organs, evidence of bloat and mild to moderate pulmonary edema. Subepicardial and myocardial hemorrhages have also been reported. There are several diagnostic aids that can assist the veterinarian in confirming an NPN poisoning.

First, assessing the rumen pH either ante-mortem or shortly after death is a very useful tool. In animals succumbing to NPN intoxication, rumen pH is typically greater than 8–10. A markedly elevated rumen pH post-mortem will gradually revert to normal over time due to continuous microbial activity post-mortem. Other causes of excessive rumen pH can include rumen impactions or other problems leading to a "dead" rumen, or excessive salivary secretions.

Elevated ammonia concentrations in various biological specimens (e.g., serum, plasma, ocular fluid and abomasal or rumen fluid) can be analytically confirmed by various methods. All samples should be collected and frozen immediately for delivery to the diagnostic laboratory, to prevent loss of the highly volatile ammonia. Samples collected post-mortem should be retrieved shortly after death; decomposition of tissues can cause either increases or decreases in ammonia levels which can muddle the interpretation. Ammonia concentrations typically greater than 1.0 mg/dl in serum or plasma and greater than 80 mg/dl in rumen or abomasum contents are diagnostically significant.

Lastly, ammonia, nitrogen, or urea levels can be assessed in the "suspect" feed source. These analyses should be quantitative, and should assist the veterinarian in determining whether a mixing error was responsible for the clinical problem.

## TREATMENT

Rapid intervention is necessary in order for affected animals to survive. This is rarely possible except in experimental settings. Poisonings generally occur on a large scale (rarely a single animal problem) and in a setting (e.g., feedlots, pastures typically located in more rural settings) where it is nearly impossible to implement appropriate rapid therapies. Treatments are generally thought to be effective if initiated within 20 min after the onset of clinical signs, and prognosis is poor for recumbent animals.

Five percent acetic acid (i.e., vinegar) should be orally infused into the affected animal; 2–6 l in cattle and 0.5–1.0 l in sheep and goats (Lloyd, 1981). This should be immediately followed by a large volume (5–10 gallons) of cold water. The acetic acid will lower the rumen pH and shift the concentrations of ammonia to the ammonium ion, which will slow down the systemic absorption of  $\text{NH}_3$ . The cold water will lower the rumen temperatures and slow down the urease enzyme, responsible for the hydrolysis of urea to ammonia. Animals that respond to this treatment may relapse. If this should occur, the drench should be cut in half. Recumbent and convulsing animals respond poorly to this treatment. Other nonspecific, supportive therapies may include anticonvulsant therapy (e.g., phenobarbital, pentobarbital) and intravenous fluid therapy to correct fluid deficits and any existing elemental or electrolyte abnormalities.

Animals that do recover from the acute insult do not appear to suffer any recognizable long-term effects, though abortions have been reported to occur in pregnant animals.

## AMMONIA

NPN poisonings resulting in hyperammonemia should not be confused with ammonia poisoning. Synonyms include ammonia gas, anhydrous ammonia and liquid ammonia. Livestock can be poisoned by ammonia, either through inhalation or ingestion, through its use as a fertilizer. Ammonia readily dissolves in water to form ammonium hydroxide. Anhydrous ammonia is colorless, highly irritating and causes corrosive injury to the mucous membranes of the eyes, lungs and skin. Inhalation of ammonia can lead to severe bronchiolar and alveolar edema, and airway destruction that can result in respiratory failure and death. Dermal contact or oral exposures lead to corrosive skin lesions and corrosive damage to the mucosal lining of the oral cavity, esophagus and stomach.

## CONCLUSIONS

All mammalian species are susceptible to urea/ammonia poisoning, although poisoning occurs with a greatest frequency in cattle by consumption of the contaminated feed. Diagnosis is based on clinical signs and analysis of the body tissues/fluids and feed for urea and ammonia levels. Treatment includes oral administration of acetic acid (vinegar) and symptomatic and supportive therapies.

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# Water quality and contaminants

Michael P. Carlson

## INTRODUCTION

Water is essential for all forms of life. The availability of adequate quantities and quality of water dictates the types of life and numbers of individuals that an environment can sustain and may be a limiting factor for livestock production systems. Less water may become available for animal production and what is available may be of poorer quality as water supplies for human use become limited, too. Large-scale animal production practices and emphasis on animal performance may increase the demands on water delivery systems and increase concern about water quality. Water quality generally refers the suitability of water for some purpose. Parameters used to assess water quality vary depending upon its proposed use and may include color, odor, taste, bacterial content, mineral content, salinity and the amounts of inorganic or organic compounds it contains.

The intention of this chapter is to provide the reader with information about water to aid in the assessment of water quality for use by companion and production animals. Some of the more readily accessible water quality standards applicable to drinking water for animals are highlighted. Detailed discussions of the mechanisms by which those chemicals cause adverse effects may be found elsewhere in this book. Information about the daily amounts of water required by animals is also provided to help assess cases of possible exposure of animals to water-borne poisons. Lastly, the risk assessment of blue-green algae poisoning is discussed.

## THE ASSESSMENT OF WATER QUALITY

Water quality for animals may be assessed for several reasons: to determine if the available water is suitable for a particular purpose, to determine if there has been a significant change in water quality, or to determine if adverse health effects that are occurring may be attributable to water contamination. Numerous commercial, governmental and academic laboratories offer water quality analytical services. The assessment of the analytical results from such laboratories requires standards against which to compare the results. National, state or provincial, or local governments may regulate water quality and water quality standards may be established by any or all of those governmental bodies. The quality of drinking water intended for consumption by humans is almost always more extensively regulated than for any other water use.

Reports of results of water quality analyses often include water quality standard ranges against which to assess the reported results. They may include graphical comparisons of the results to the water quality standards, making identification of parameters that fall outside of the standard limits easily identifiable. Such reports often use water quality standards intended for human drinking water, so the user of the report must also assess the suitability of the reported standards for the intended use of the water.

Assessment of the results of water quality analyses must be done using standards that are applicable for the intended use of the water and which are in force for the venue at which the water is to be used. The suitability of



water intended for use as drinking water by animals is better assessed using drinking water standards for animals instead of standards for humans. For example, the U.S. human drinking water standard for nitrate + nitrite is  $10 \text{ mg NO}_3^- + \text{NO}_2^- - \text{N/L}$  (U.S. EPA, 2009a), whereas the livestock Canadian standard for nitrate + nitrite is  $100 \text{ mg NO}_3^- + \text{NO}_2^- - \text{N/L}$  (CCME, 2005a,b). That 10-fold difference can have a significant effect on water quality assessment. Animal owners may take unnecessary and costly actions to mitigate what are erroneously believed to be unacceptably high risk of some sort of adverse health effect.

Raisbeck *et al.* (2011) list several factors that need to be considered when assessing water quality for livestock or wildlife if the following question is posed by an animal owner or manager: "What will <chemical name> present in the water at <concentration> do to my animals?"

- 1 Many chemicals in water interact additively with the same chemicals in feedstuffs. In such circumstances, it is not the water concentration that is singly important but the total dietary content for those chemicals. For example, nitrate ingested from forage is usually much greater than that ingested from water.
- 2 Water quality analytical methods do not necessarily measure the specific chemical form of the chemical present in the water. For example, selenium is usually reported as total selenium and not as the chemical form present, such as selenite or selenate.
- 3 Reports of water quality do not usually differentiate between animal species. The toxicity of chemicals can differ significantly between species. This point has already been highlighted in the previous paragraph of this chapter.
- 4 Many chemical substances in the diet interact with each other. Those chemicals may be either nutrients or potential toxicants. For example, the absorption of copper depends upon dietary molybdenum and sulfur-containing compounds.
- 5 Rate of exposure influences the rate at which adverse health effects may become evident. For example, the ingestion of nitrate-fertilizer-contaminated water usually produces adverse effects much more rapidly than does ingestion of high-nitrate-containing fresh forage.

## WATER QUALITY STANDARDS FOR ANIMALS

Water quality standards for animals are not the same as nor are they enforced with the same authority as those for humans. Water quality recommendations for animals

have been made by the U.S. EPA in 1973 (U.S. EPA, 1973), the National Academy of Sciences (NAS) in 1974 (NAS, 1974) and Canada (CCME, 2005a,b). Table 99.1 lists water quality recommendations for livestock taken from each of those sources for selected chemicals.

Those publications include recommendations for water use by animals other than livestock. Recommendations for other animals are listed in Table 99.2. Readers may find them to be more applicable than the livestock recommendations in certain circumstances.

### U.S. EPA water quality criteria for animals

The U.S. EPA published proposed water quality standards for irrigation, livestock, aquatic life, wildlife, public freshwater, marine aquatic life and recreational water in 1973 (U.S. EPA, 1973). The criteria were formulated and published by the U.S. EPA pursuant to the Federal Water Pollution Control Act Amendments of 1972 and the Water Quality Act of 1965. The report states: "Almost all of the criteria are taken from the recommendations of the National Academy of Science's report on Water Quality Criteria (in press) developed under contract to the Environmental Protection Agency." Those recommendations do not appear to have been revised since their publication.

### National Academy of Sciences recommended limits in drinking water for livestock and poultry

The NAS report published in 1974 (NAS, 1974) summarizes what was known at the time of publication about effects of nutrient and toxic substances that were found in water consumed by domesticated animals. It also contains information about water requirements and the percentages of recommended intake of various substances provided by normal daily water consumption, and toxic concentrations for various species. The publication includes a table summarizing the effects of toxic concentrations of various chemicals in water for various domestic and laboratory animals. The information included in those tables is too extensive to reproduce in this chapter. Readers are urged to consult them as needed.

An ad hoc committee of the National Research Council's Committee on Animal Nutrition reviewed the scientific literature related to minerals and toxic substances in the diets and water for animals. Its findings and recommendations were published as a second revision of mineral tolerances of animals (Committee on Minerals, 2005). Drinking water standards cited in that revision still refer to the 1974 NAS publication (NAS, 1974).

TABLE 99.1 Water quality recommendations for selected chemicals for livestock

Chemical	Upper concentration limit (mg/L)			Microorganisms	U.S. EPA (1973)	NAS (1974)	CCME (2005a)
	U.S. EPA (1973)	NAS (1974)	Canadian (CCME, 2005a)				
Arsenic (As)	0.2	0.2	0.025	Cyanobacteria (blue-green algae)	Heavy growth of blue-green algae not acceptable	–	No water from water sources that contain heavy growths of blue-green algae, especially if there is a history of blue-green algal toxicosis for the water source
Cadmium (Cd)	0.050	0.05	0.080	Microorganisms	5000 coliforms/dL (average of $\geq 2$ samples/month), 20,000/dL (individual sample)	–	–
Calcium (Ca)	–	–	1000	Fecal coliforms	1000/dL (average of $\geq 2$ samples/month), 4000/dL (individual sample)	–	–
Chloride ( $\text{Cl}^-$ )	–	–	No data	Water-borne pathogens	–	–	No data
Chromium (Cr)	1.0	1.0	No data				
Total							
$\text{Cr}^{\text{III}}$			0.050				
$\text{Cr}^{\text{VI}}$			0.050				
Copper (Cu)	0.5	0.5	1.0 (cattle), 5.0 (swine, poultry), 0.5 (sheep)				
Cyanide ( $\text{CN}^-$ )	–	–	No data				
Fluoride ( $\text{F}^-$ )	2.0	2.0	2.0 (if none in feed) 1.0 (if also in feed)				
Hardness	–	–	–				
Iron (Fe)	No limit	No limit	No data				
Lead (Pb)	0.1	0.1	0.1				
Manganese (Mn)	No limit	No limit	No data				
Mercury (Hb)	1.0	0.010	0.003				
inorganic organic							
Nitrate ( $\text{NO}_3^-$ )	–		No data				
(as N)		100					
(as $\text{NO}_3^-$ )		440					
Nitrate + nitrite							
( $\text{NO}_3^- + \text{NO}_2^-$ )	23 ( $\text{NO}_3^- + \text{NO}_2^-$ )	–	100 ( $\text{NO}_3^- + \text{NO}_2^-$ )				
(as N)	100 ( $\text{NO}_3^- + \text{NO}_2^-$ )	–	440 ( $\text{NO}_3^- + \text{NO}_2^-$ )				
(as $\text{NO}_3^-$ )							
Nitrite ( $\text{NO}_2^-$ )							
(as N)	3.0	10	10				
(as $\text{NO}_2^-$ )	10	33	33				
Selenium (Se)	0.05	–	0.050				
Sulfate ( $\text{SO}_4^{2-}$ )	–	–	1000				
Total dissolved solids	–	–	3000				
(TDS) (salinity)							
Zinc (Zn)	25	25.0	50				

TABLE 99.2 Water quality recommendations for selected chemicals for aquatic water systems

Water system	Freshwater aquatic life		Freshwater wildlife	Marine aquatic life	
Agency	CCME (2005b)	U.S. EPA (1973)	U.S. EPA (1973)	CCME (2005b)	U.S. EPA* (1973)
Chemical	Upper concentration limit (mg/L)				
Arsenic (As)	0.005	—	—	0.0125	0.01 × 96 hr LC <sub>50</sub> ; 0.5
Cadmium (Cd)	$[Cd] = \{10^{0.86[\log_{10}(\text{hardness})] - 3.2}\} / 1000$ ; units = mg/L** Hardness as CaCO <sub>3</sub>	0.03 in hard water, 0.004 in soft water	—	0.00012	0.01 × 96-hr LC <sub>50</sub> ; 0.01 mg/L
Calcium (Ca)	No data	—	—	No data	—
Chloride (Cl <sup>-</sup> )	No data	—	—	No data	—
Chromium (Cr)					0.01 × 96-hr LC <sub>50</sub> ; 0.1 mg/L
Total	No data	0.03	—	No data	
Cr <sup>III</sup>	0.0089			0.056	
Cr <sup>VI</sup>	0.001			0.0015	
Copper (Cu)	Minimum of 0.002 regardless of water hardness $[Cu] = \{e^{0.8545[\ln(\text{hardness})] - 1.465}\} / 1000$ ; units = mg/L** Hardness as CaCO <sub>3</sub>	0.1 96-hr LC <sub>50</sub>	—	No data	0.01 × 96-hr LC <sub>50</sub> ; 0.05 mg/L
Cyanide (CN <sup>-</sup> )	0.005 (as free CN <sup>-</sup> )	0.05 96-hr LC <sub>50</sub>	—	No data	0.1 × 96-hr LC <sub>50</sub> ; 0.01 mg/L
Fluoride (F <sup>-</sup> )	No data	—	—	No data	0.1 × 96-hr LC <sub>50</sub> ; 1.5 mg/L
Hardness	—	See TDS	—	—	—
Iron (Fe)	—	—	—	No data	0.3
Lead (Pb)	Minimum of 1 µg/L regardless of water hardness $[Pb] = \{e^{1.273[\ln(\text{hardness})] - 4.705}\} / 1000$ ; units = mg/L** Hardness as CaCO <sub>3</sub>	0.03	—	No data	0.02 × 96-hr LC <sub>50</sub> ; 0.1 qLD <sub>50</sub> (sic)
Manganese (Mn)	No data	—	—	No data	0.02 × 96-hr LC <sub>50</sub> ; 0.01 mg/L
Mercury (Hg)		Inorganic:			
Inorganic	No data	0.0002 (total)	0.5 mg/kg in fish	No data	0.01 × 96-hr LC <sub>50</sub> ; 0.1 mg/L
Organic	0.000004	0.00005 (average) 0.05 mg/kg (total body burden) Organic: same as inorganic			
Nitrate (NO <sub>3</sub> <sup>-</sup> ) (as N)	2.94	—	—	3.61	—
(as NO <sub>3</sub> <sup>-</sup> )	13			16	
Nitrate + nitrite (NO <sub>3</sub> <sup>-</sup> + NO <sub>2</sub> <sup>-</sup> ) (as N)	No data	—	—	No data	—
(as NO <sub>3</sub> <sup>-</sup> )					
Nitrite (NO <sub>2</sub> <sup>-</sup> ) (as N)	0.06	—	—	No data	—
(as NO <sub>2</sub> <sup>-</sup> )					
Selenium	0.001	—	—	No data	0.01 × 96-hr LC <sub>50</sub> ; 0.01 mg/L
Sulfate	No data	—	—	No data	—
Total dissolved solids	No data	Bioassays	—	No data	—
Zinc	0.030	0.003 × 96-hr LC <sub>50</sub>	—	No data	0.01 × 96-hr LC <sub>50</sub> ; 0.1 mg/L
<b>Microorganism</b>					
Cyanobacteria (blue-green algae)	No data	—	No limit	—	—
Microorganisms	—	—	—	—	—
Fecal coliforms	—	—	—	—	—
Water-borne pathogens	No data	—	—	No data	—

\*Recommended concentration limits are expressed as the fraction of either lethal concentration – 50 (LC<sub>50</sub>) – or lethal dose – 50 (LD<sub>50</sub>). The concentration listed thereafter is the maximum concentration considered acceptable.

\*\*Equation from reference calculates concentration in units of µg/L; dividing that concentration by 1000 gives concentration in units of mg/L; website has capability of calculating concentration with input of water hardness as CaCO<sub>3</sub>.

## Canadian environmental quality guidelines

Canadian water quality standards for agricultural use, including livestock, were first published in a document titled *Canadian Water Quality Guidelines* in 1987 by the Canadian Council of Resource and Environment Ministers (CCREM). That organization was later renamed the Canadian Council of Ministers of the Environment (CCME). In April 1996, CCME began work that consolidated national environmental quality guidelines for water, soil, sediment, tissue residues and air into one document, which was published as the *Canadian Environmental Quality Guidelines* by CCME. The guidelines are continually reviewed and updated as deemed necessary.

Water quality standards have continued to be revised and current information is readily available on the CCME Canadian Environmental Guidelines Summary Table website at <http://st-ts.ccme.ca/>. The site is designed to produce a listing of the chemicals and the guidelines of interest to the user.

## Organic contaminants

There are numerous organic chemicals, including many used in modern agricultural production, that are also included in various water quality tables. Such chemicals do not seem to be an issue for the quality of water for animals.

Drinking water quality does not appear to be an issue with organic production standards. The USDA National Organic Program's Organic Production and Handling Standards (USDA, 2002) do not include water quality among its livestock standards. The standards contain the following about water (bold font added to identify the mention of water):

Year-round access for all animals to the outdoors, shade, shelter, exercise areas, fresh air, **clean water for drinking**, and direct sunlight, suitable to the species, its stage of life, the climate, and the environment: Except, that, animals may be temporarily denied access to the outdoors in accordance with §§205.239(b) and (c).

European Union legislation has the following mention of water quality (bold font added to identify the mention of water) (Council Regulation 834/2007):

husbandry practices, including feeding, design of installations, stocking densities and **water quality** shall ensure that the developmental, physiological and behavioral needs of animals are met;

## DRINKING WATER QUALITY STANDARDS FOR HUMANS

Drinking water quality standards for selected chemicals that are published by the U.S. EPA (U.S. EPA, 2009a,b,c,d), Canada (Health Canada, 2010) and the World Health Organization (WHO, 2004) are listed in Table 99.3. They are included in this chapter to allow readers to compare standards for animals and humans. The adverse health effects listed in U.S. EPA documents for each chemical are also listed in the table.

In the U.S., legal limits called maximum contaminant levels (MCLs) have been established for about 90 contaminants. If contaminant concentrations in public water supplies and certain private supplies are found to be above applicable MCLs, then the water supplier must take action to bring the contaminant concentration down below the MCL. Meanwhile, an alternate source of water that meets the standards must be provided.

In the U.S., there are primary and secondary water standards for human drinking water (U.S. EPA, 2009b,c). Primary drinking water standards are legally enforceable and water providers cannot provide water containing contaminants higher than their respective MCLs. Secondary standards are non-enforceable guidelines for contaminants that may cause cosmetic effects, such as skin or tooth discoloration, or unacceptable aesthetic effects, such as unacceptable taste or odor from or discoloration of the water.

Primary standards are established considering the adverse health effect caused by the contaminant and

TABLE 99.3 Water quality recommendations for human drinking water: selected contaminants

Agency	Canadian			Potential health effects from exposure above maximum acceptable concentration (U.S. EPA, 2005b)
	U.S. EPA (2005a)	(Health Canada, 2010)	WHO (2004)	
Chemical	Maximum acceptable concentration (mg/L)			
Arsenic	0.010	0.01	0.01 (provisional)	Increased risk of cancer
Cadmium	0.005	0.005	0.003	Kidney damage
Calcium	—	—	—	—

(Continued)



TABLE 99.3 (Continued)

Agency	U.S. EPA (2005a)	Canadian (Health Canada, 2010)	WHO (2004)	Potential health effects from exposure above maximum acceptable concentration (U.S. EPA, 2005b)
Chemical	Maximum acceptable concentration (mg/L)			
Chloride	250 (secondary)	≤250 (aesthetic objective)	No health concerns at usual concentrations	–
Chromium	0.1 (total)	0.005	0.05 (provisional)	Allergic dermatitis
Copper	1.3 (at tap)	≤1.0 (aesthetic objective)	2	Short-term exposure: gastrointestinal distress Long-term exposure: liver or kidney damage; in cases of Wilson's disease consult physician if water Cu concentration exceeds MCL
Cyanobacterial toxins (microcystin-LR)	–	0.0015	–	
Fluoride	4.0	1.5	1.5	Bone disease; pain and tenderness of the bones; mottled teeth in children
Hardness	–	–	–	–
Iron	0.3 (secondary)	≤0.3 (aesthetic objective)	No health concerns at usual concentrations	–
Lead	0.015 (at tap)	0.010 (thoroughly flush faucet before water is taken for consumption or analysis)	0.01	Infants and children: physical or mental development delays, slight attention span deficits and learning disabilities
Mercury	0.002 (inorganic)	0.001	0.001 (total)	Adults: kidney problems, hypertension Kidney damage
Nitrate (as N)	10	10	–	Infants <6 months old: blue-baby syndrome
(as NO <sub>3</sub> <sup>-</sup> )	45	45	50 (short term)	
Nitrite (as N)	1	–	–	Infants <6 months old: blue-baby syndrome
(as NO <sub>2</sub> <sup>-</sup> )	3.3	<3.2, where nitrite is measured in addition to nitrate		
Selenium	0.05	0.01	0.01	Hair or fingernail loss; numbness in extremities; circulatory problems
Sulfate	250 (secondary)	≤500 (aesthetic objective)	No health concerns at usual concentrations	–
Total dissolved solids	500 (secondary)	≤500 (aesthetic objective)	No health concerns at usual concentrations	–
Zinc	5 (secondary)	≤5.0 (aesthetic objective)	No health concerns at usual concentrations	–
Microbes	U.S. EPA	Canadian	WHO	
Total coliforms	≤5% positive samples in a month; positives must be analyzed for fecal coliforms	0 per 100 mL		Presence indicates that potentially harmful bacteria may be present; see fecal coliforms, <i>E. coli</i>
Fecal coliforms	Repeat positive sample is considered in violation	–		Presence indicates water may be contaminated with human or animal wastes; short-term effect: diarrhea, cramps, nausea, headaches, or other symptoms; special health risks for infants, young children, people with severely compromised immune system
<i>E. coli</i>	Repeat positive sample is considered in violation	0 per 100 mL		See fecal coliforms

the dose at which such effects occur. A reference dose (RFD or RfD) is estimated based upon the amount of the contaminant to which a person may be exposed *on a daily basis that is not anticipated to cause the adverse health effect over a person's lifetime*. Consequently, the standards may not reflect the risk of adverse health effects in animals, and care should be used applying them to water intended for consumption by animals. A complete listing of drinking water standards and health advisories applicable to humans is compiled as needed by the U.S. EPA Office of Water. The latest available at the time this chapter was written was published in 2009 and may be accessed on the U.S. EPA website (U.S. EPA, 2009a).

## WATER CONSUMPTION TABLES

The dose of a water-borne contaminate may have to be estimated in cases of suspected toxicoses and may be calculated if the contaminant's concentration in the water and the amount of water consumed by the victims are known. Contaminant concentration in the water may be determined by analysis, but water consumption by the victims is rarely known with certainty.

Water consumption varies by species, weather condition, diet and state of health. All of those factors and their interactions make the determination or estimation of minimal water requirements difficult if not impossible. Additionally, daily water requirements are not necessarily met solely by drinking water consumption.

Use water consumption data to calculate doses of water-borne poisons, if such data are available. If not, use the data in Table 99.4, which was taken from the NAS publication (NAS, 1974), to estimate water consumption. Those data should be modified for situations falling outside of the defining parameters listed in the title.

**TABLE 99.4** Anticipated water consumption of various adult animals of medium weight in temperate climates

Animal	Expected consumption (L/day)
Beef cattle	26–66
Dairy cattle	38–110
Horses	30–45
Swine	11–19
Sheep, goats	4–15
Chickens	0.2–0.4
Turkeys	0.4–0.6

Data are from NAS (1974).

## RATIONALE FOR LIVESTOCK RECOMMENDATIONS

The rationales for the chemicals included in Table 99.1 livestock recommendations included in the U.S. EPA Proposed Criteria for Water Quality Volume 1 (U.S. EPA, 1973) are summarized below. Information listed was what was known at the time the recommendations were formulated and may not reflect current knowledge of the effects of the chemicals. Our addenda are so noted.

The EPA writes in its introduction to the document:

Acceptable limits specified in the recommendations were derived by the application of scientific judgment to lethal dose or lethal concentration data in a manner that provides a margin of safety to test organisms. For those substances whose effects are more aptly described as undesirable such as impairing aquatic habitats, causing taste and odor problems in water supplies, or reducing the aesthetic or recreational quality of a water body, limits which minimize these effects were established on the basis of field and laboratory investigations. Acceptable levels of toxic materials for which specific numerical maximum acceptable concentrations are not prescribed are determined by applying an application factor to locally derived LC<sub>50</sub> data. By basing criteria on effects on the most sensitive important species, a desirable degree of regional and local variation is introduced, allowing water quality standards to depend on local conditions.

An "important species" in the criteria is defined as an organism that: (1) is commercially or recreationally valuable; (2) is rare or endangered; (3) affects the well-being of some species within (1) and (2); or (4) is critical to the structure and function of the ecological system. A "rare or endangered" species is any species so officially designated by the U.S. Fish and Wildlife Service.

### Arsenic

Toxicity of arsenic depends upon its chemical form, relating primarily to rate of excretion. Inorganic oxides are generally more toxic than are organic forms of arsenic. Acute toxicities for farm animals are given in Table 99.5.

Arsenic acid fed to lactating cows at up to 1.25 mg/kg body weight for 8 weeks, equivalent to an intake of 60 L of water containing 5.5 mg arsenic/L daily by a 500 kg animal, was absorbed and rapidly excreted in the urine with no increase of arsenic content of milk. No toxicity was observed.

TABLE 99.5 Acute toxicity of inorganic arsenic by species

Species	Toxicity (g/animal)
Poultry	0.05–0.10
Swine	0.15–1.0
Sheep and goats	10.0–15.0
Horses	10.0–15.0
Cattle	15.0–30.0

Wadsworth 1952 as cited by U.S. EPA (1973).

TABLE 99.6 Effects of cadmium in various species

Species	Amount and source	Effect
Human	15 mg/L in popsicles	Sickening
Male rats	4.5 mg Cd/kg bw	Permanent sterility
Rats or mice	5 mg/L in drinking water	Reduced longevity
Pregnant hamsters	2 mg/kg bw of CdSO <sub>4</sub> by i.v. injection	Dose on day 8 of gestation caused fetal malformations

Data are from U.S. EPA (1973).

Cadmium

Effects of cadmium in various species are listed in Table 99.6.

A small fraction of cadmium is absorbed in ruminants, with most of what is absorbed going to the kidneys and liver. The cow is “found to be very efficient in keeping cadmium out of its milk.” Most major animal products, including beef and milk, seem “quite well protected against cadmium accumulation.”

Chromium

Chromium is not readily absorbed by animals. Most of what is ingested is excreted in the feces. It does not appear to concentrate in mammalian tissue nor does its concentration increase with age. It is concluded that up to 5 mg/L of chromium II or VI in drinking water should not be harmful to livestock. That level may be unnecessarily high and the 1.0 mg/L level is recommended to provide a “suitable margin of safety.”

Copper

Copper is an essential trace element, so some is required in the diet to maintain good health. Swine appear to be more tolerant to copper with dietary concentrations of 250 mg/kg or higher improving live weight gains and feed efficiency. Copper does not appear to accumulate in tissues. But in sheep, copper accumulates in the liver, followed by its sudden release in circulation causing hemolysis. Therefore, sheep are very susceptible to copper toxicosis.

A diet containing 25 mg/kg fed to sheep is considered toxic. About 9 mg per animal per day is considered safe.

Fluorine (Author’s addendum: fluorine and fluoride seem to be used synonymously)

Consumption of drinking water with 2.0 mg/L may produce some tooth mottling, but it is not excessive with respect to animal health or deposition in meat, milk or eggs. Chronic fluorosis of livestock has occurred with water content of 10–15 mg fluoride/L. Total ration content of 30–50 mg fluoride/L (sic) for dairy cattle is considered a safe upper limit. Transfer to milk occurs to a very small extent and to a greater degree in eggs. Fluoride at 1.0 mg/L in drinking water does not harm livestock.

Hardness (Authors’ addendum)

Hard water does not appear to have a deleterious effect on animals, but it often is reported as part of water quality analysis. Hardness is a measure of the calcium and magnesium ions present in the drinking water. Water with high TDS or salinity may or may not be hard water. Hardness of water may be measured as grains/gallon or in ppm. One grain per gallon is equivalent to 17 mg calcium and magnesium per liter. Water is classified according to calcium and magnesium salt content as follows: soft water ranges from 0 to 60 ppm, hard water varies from 120 to 180 ppm and very hard water is greater than 180 ppm.

Iron

In small quantity, iron is essential to animal life, while in excessive quantity it is considered toxic. Due to its involvement in Fenton reaction, iron is known to produce excessive free radicals causing oxidative stress. Brain, lungs and liver are the major sites of iron toxicity. Maximum recommended levels of iron in water for humans, livestock and poultry are <0.3, <0.4 and <0.4 ppm, respectively. Maximum tolerable concentrations of dietary iron have been set at 500 mg/kg for cattle, sheep and poultry, and 300 mg/kg for swine. Elevated dietary iron may antagonize copper, manganese and sulfur in lactating dairy cattle.

Lead

The toxicity of lead has not been clearly established quantitatively. Daily intake of 6–7 mg/kg body weight had been suggested as a threshold dosage in cattle, but that is

difficult to establish. A concentration of 0.5mg/L in drinking water is considered safe. Drinking water containing 5mg/L consumed by rats and mice over the course of their lifetimes produced toxic effects. Death rates of older animals increased, especially in males. Death was not caused by overt lead toxicosis, but was due to an increased susceptibility to "spontaneous infections."

## Mercury

Mercury content in surface water was found to usually be <5µg/L, but methylation of mercury in bottom sediments bordering mercury deposits resulted in the continuous presence of mercury in solution. The relative stability of methylmercury and its high absorption from the gut contributes to its oral toxicity. It had been suggested that livestock blood and tissue lead content be kept below 0.1 and 0.5mg/kg, respectively, to protect humans who might consume those tissues. The safe contamination level for fish consumed by humans had been set at 0.5mg/kg by the U.S. FDA. However, that did not take into consideration other possible sources of dietary mercury. "In view of these facts the limits prescribed herein are reduced by a factor of ten to reduce the significance of levels from meat products in comparison with those of fish" (U.S. EPA, 1973).

## Nitrate and nitrite

Nitrite is more toxic to livestock than is nitrate. Usually nitrite is produced by the reduction of nitrate to nitrite, which occurs in the rumen; in moistened feeds, such as freshly chopped green forage; or water containing sufficient organic matter to sustain microbes. Natural waters may contain "high levels" of nitrate, but they usually contain very low concentrations of nitrite. Nitrate in cattle feed did not seem to pose a hazard to humans consuming dairy products or meat produced from cattle eating the nitrate-containing feed. "Animals fed nitrate continuously develop some degree of adaptation (sic) to it." "Assuming maximum water consumption by dairy cattle of 3–4 times the dry matter intake, the concentration of nitrate to be tolerated in water should be about one fourth of that tolerated in the feed. This would amount to about 300mg/l of nitrate." Effects of nitrate and nitrite at various concentrations given to non-ruminants are summarized in Table 99.7.

It appeared that ingestion of water containing up to 300mg/L nitrate or 100mg/L nitrite was well tolerated by all classes of livestock and poultry studied under controlled experimental conditions.

Authors' addendum: Crowley *et al.* (1974) conducted a 35-month study in Wisconsin comparing reproductive

efficiency and lactational performance for a 54 cow Holstein herd that consumed drinking water containing either 19 or 374ppm nitrate. During the last 15 months of the study, cattle drinking the elevated nitrate containing water had the highest services per conception and lowest first service conception rates. The average milk yield was not significantly different between the two groups but the total milk yield for the entire 35-month study was lower in the elevated water nitrate group. This small difference in milk yield in the elevated nitrate containing drinking water was thought to be due to an increased dry period due to lower conception rates.

The author has never encountered a case of acute nitrate or nitrite toxicosis due to the consumption of drinking water, unless the water had been hauled to the animals in a fertilizer tank. Such cases have always involved cattle and occur when the availability of drinking water is disrupted and drinking water must be hauled to the animals from elsewhere. Such circumstances may occur when water freezes during stretches of very cold weather or the water pump fails and cannot be rapidly repaired. Deaths often occur relatively rapidly; owners often report finding dead victims within a few meters of the contaminated water tank. Ocular fluid collected from dead victims usually contains nitrate in excess of 100µg nitrate/mL and nitrite in excess of 2µg nitrite/mL. Ocular fluid collected from dead victims of forage nitrate toxicosis usually contains less nitrate than ocular fluid from dead victims of water nitrate toxicosis. If the fertilizer tank contained ammonium nitrate, then death may be the result of either excessive ammonia or nitrate exposure, and the determination of the exact cause of death is mostly academic. Under no circumstances do we recommend that a tank that has contained fertilizer be used to haul drinking water to animals, even if it has been "thoroughly washed out." Theoretically, a

TABLE 99.7 Effects of nitrate and nitrite administered to non-ruminants

Species	Concentration, dosage or dose	Observed effect(s)
Gilts from weaning through two farrowing seasons	330mg/L (nitrate)	No adverse effects
Growing pigs	330mg/L (nitrate)	No adverse effects
Chicks and laying hens	Up to 300mg/L (nitrite)	No effects on growth or production
Chicks and laying hens	Up to 200mg/L (nitrite)	No effects on growth or production
Turkey pullets	200mg/L (nitrite)	Decreased growth
Turkeys, laying hens and turkeys	200mg/L (nitrite)	Reduced liver vitamin A stores
	50mg/L (nitrite)	No adverse effects observed

Data are from U.S. EPA (1973).



fertilizer tank may be cleaned sufficiently to haul drinking water, but not practically.

Total dissolved solids (TDS)

“Total dissolved solids” is defined in the NAS publication (NAS, 1974) as the concentration of all dissolved constituents in water. “Salinity” is often used synonymously with “total dissolved solids,” although that assumes that all of the dissolved solids are saline. Salinity is more than a measurement of the total amount of sodium and chloride present in water. Salinity also includes anions such as carbonates expressed as oxides, bromide and iodine expressed as chlorine, and cations such as calcium, magnesium, bicarbonate and sulfate, but does not include organic matter.

The U.S. EPA does not include recommendations for TDS, but the NAS provides criteria for classification of water based upon TDS content established for the U.S. Geological Society. Criteria for two classification systems are listed in Table 99.8. Note the differences in the classification and the use of brine in both, but with different criteria for inclusion in that classification. It has been our experience that those classifications contribute little to the assessment of water quality for animals as we have never encountered an instance where those water classifications have been used to describe the TDS content. Nevertheless, they are included here. More useful information about the use of saline waters for livestock is provided under the section titled “A Guide to the Use of Saline Waters for Livestock” in the NAS publication, starting on p. 48 (NAS, 1974).

Several factors need to be considered in assessing the suitability of saline water for livestock use, including kind, age and sex of the animals; pregnancy or lactation status; physical exertion; climatic conditions;

TABLE 99.8 Water classifications based upon total dissolved solid content

DeWiest classification (1966)		Robinove <i>et al.</i> classification (1958)	
Water classification	TDS content (mg/L)	Water classification	TDS content (mg/l)
Fresh water	<1000		
Brackish water	1000–10,000	Slightly saline	1000–3000
		Moderately saline	3000–10,000
Salty water	10,000–100,000	Very saline	10,000–35,000
		Brine	>35,000
Brine	>100,000		

Data are from U.S. EPA (1974).

diet and its moisture and mineral content; production expectations; water salt content; access to other water sources; and the adaptation to available water sources. Weight given to those factors is largely a matter of judgment, but TDS is the single most reliable factor that can be determined.

The NAS did not recommend the use of highly saline water for livestock but acknowledged that circumstances may arise such that saline water may be all that is readily available. Table 99.9 lists the guidelines for use of saline water for livestock and poultry. If high-TDS water is to be used, the following points should be considered, too:

- 1 Alkalinities and nitrate should be considered whenever water containing >3000mg TDS/L is to be used. Alkalinities of 2000mg CaCO<sub>3</sub> detract from the suitability of water. Hydroxide is more harmful than carbonate, which is more harmful than bicarbonate.

TABLE 99.9 Guide to use of saline waters for livestock and poultry

Water TDS content (mg/L)	Expected health or performance effects
< 1000	None expected for any livestock class
1000–2999	None expected for any livestock class. May cause temporary diarrhea or watery droppings in animals unaccustomed to the water
3000–4999	Should be none. May cause temporary diarrhea or be refused at first by animals unaccustomed to the water. Poor water for poultry, often causing watery droppings, increased mortality and decreased growth, especially in turkeys
5000–6999	Reasonably safe for dairy and beef cattle, sheep, swine and horses. Avoid use of water approaching the higher limit for pregnant or lactating animals. Unacceptable for poultry; use for poultry will almost always be accompanied by some type of problem, especially near the upper limit, such as reduced growth or production
7000–10,000	Unfit for poultry and probably swine. Considerable risk may exist for pregnant or lactating cows, horses, sheep and the young of those species. Considerable risk for animals subject to heavy heat stress or water loss. Generally use of such water should be avoided, although older ruminants, horses and even poultry and swine may subsist on it for long periods of low stress
>10,000	Unacceptable risks for any livestock class

Taken from NAS (1974).

- 2 If offered the choice between two water sources, one highly saline and one less saline, they will choose the less saline source.
- 3 Animals can tolerate high saline water for a few days if they are then given low saline water.
- 4 Water intake usually increases with soluble salt content, except in cases of extremely high saline content, which animals may refuse to drink.
- 5 Abrupt changes from a low to high saline water will likely cause more problems than a gradual change.
- 6 Decreased water intake is very likely accompanied by decreased feed intake. So, animals with high production expectations may not produce as well showing "deleterious effects from waters of lower salts content than animals on a maintenance regimen."
- 7 Highly saline water may furnish enough minerals to be considered in dietary mineral formulations. And salt content of the diet may contribute to the toxicity of saline water, particularly if salt is added to control feed intake. Generally, water should not normally be relied upon as a source on essential inorganic minerals.

## Selenium

At the time that the U.S. EPA formulated its recommendations, no substantiated case of livestock poisoning by selenium in waters had been found. However, selenium toxicosis was found in wildlife exposed to high selenium-containing irrigation runoff water. Agricultural drainage water entering Kesterson Reservoir in California ranged from 0.140 to 1.40 mg/L (140 to 1400 ppb) during 1983–1985.

## Sulfate

No recommendation for sulfate was made by the U.S. EPA probably because the association between sulfate exposure and polioencephalomalacia in ruminants had not yet been discovered. The Canadian recommendation is 1000 mg/L. Exposure to elevated sulfates in drinking water can result in a transient diarrhea. Animals usually acclimate to an elevated level of drinking water sulfate in 3 to 7 days and no longer exhibit diarrhea. Methemoglobin was increased 450% in cattle consuming drinking water containing sulfates at 3493 ppm.

## Zinc

Zinc is relatively less toxic. The findings cited in the EPA document are summarized in Table 99.10. Increased zinc intake increased the zinc content of body tissue, but its accumulation was not great and tissue content fell rapidly after zinc intake was reduced.

## HEALTH EFFECTS OF CYANOTOXINS FROM WATER

The effects of cyanotoxins from water on humans are discussed by Kuiper-Goodman *et al.* (1999) and serve as the basis for risk assessment for exposure from drinking and recreational water by the World Health Organization (WHO, 2004). Relevant information from their chapter is summarized here. A more detailed discussion of cyanotoxins may be found elsewhere in this book (Chapter 72).

## Cyanotoxins

Toxic effects after exposure to cyanobacteria are evidenced by retrospective epidemiological studies of human disease, factual and anecdotal reports of disease in animals, and toxicological studies of cyanotoxins. Cyclic peptides, like microcystins, are specifically toxic to the liver in mammals and of particular concern to human health. Acute toxicoses cause liver hemorrhage and death from liver failure. Chronic toxicoses may promote liver and other cancers.

Alkaloid neurotoxins, like anatoxins and saxitoxins/paralytic shellfish poison (PSP), have shown only acute toxicoses in mammals. Information about anatoxins is sparse. Some of the alkaloid toxins, especially PSP, accumulate in marine and freshwater biota, which may place animals and humans consuming such biota at risk of exposure after consuming them.

Cyanobacterial lipopolysaccharides (LPS) can elicit allergic and toxic responses in humans, although little is known about their acute or chronic effects. They should be considered whenever gastrointestinal and respiratory signs are reported or observed.

## Adverse health effects in humans

Drinking water has been associated with gastrointestinal (GI) illness throughout history. Pathogenic bacteria and viruses are common causes of such illness, and evidence for GI illness caused by cyanotoxin exposure needs to

TABLE 99.10 Zinc toxicity in diets for various animals

Dietary per water Zn content	Species and effect(s)
1000 mg/kg in diet; >2000 2320 mg/L in water	Swine tolerated it; Swine: toxic Chickens: reduced water consumption, egg production, body weight
>500 mg/kg in diet, as oxide	Ruminants: toxic

Data are from U.S. EPA (1973).

take such etiologies into account. Cases of GI and hepatic illness that can be reliably attributed to cyanotoxins have all been coincidental with lysis of cyanobacterial blooms, either natural or artificial. Lysis releases cyanotoxins from the cells into the water.

Artificial lysis occurs after water treatment with copper sulfate to destroy blooms in water reservoirs, and poisonings subsequent to such treatment have been reported in the U.S. and Australia. Water filtration after the treatment can remove cell fragments, but not dissolved cyanotoxins.

Individuals at greater risk of cyanotoxicoses are the young, who drink a higher volume of water than adults in proportion to their body weights, and subjects with liver disease or kidney damage. Chronic effects in humans are of great concern because of the potential of exposure to low concentrations of cyanotoxins in drinking water over the course of a lifetime. Results of animal experiments have indicated chronic exposure to microcystins may promote tumor growth.

Epidemiological studies of the incidence of human hepatocellular carcinoma in China have identified three risk factors: hepatitis B viral infection, exposure to aflatoxin B<sub>1</sub> in food and the source of drinking water. Cancer mortality is lower when the source of drinking water is from deep well than when it is from surface waters. Cyanobacteria are abundant in the same areas where the incidence of hepatic carcinoma is highest.

There have been repeated reports of adverse effects in swimmers exposed to cyanobacterial blooms, such as skin irritation and GI symptoms. Deep blisters on skin under bathing suits have been reported, particularly after contact with the marine cyanobacterium *Lyngbya majuscula*, which is believed to occur when the organism becomes trapped against the skin under the bathing suit. Effects may be due to allergic reactions to cyanobacterial pigments or direct contact with the cyanotoxins. Individual sensitivity to cyanotoxins varies significantly.

Health effects in humans exposed to cyanobacteria in recreational waters include headache, nausea, vomiting, muscular pain, painful diarrhea, abdominal pain, blistering of the lips, sore throats, skin rashes, fever and eye or ear irritation, usually within 7 days of exposure. Severity of symptoms appears to be related to full-body immersion, the amount of water ingested, and the duration of exposure. Severe pneumonia attributed to aspiration of a *Microcystis* toxin was reported to have occurred in two British army recruits after they swam in and underwent canoe training in water containing a dense bloom of *Microcystis* spp.

## Effects in animals

Deaths due to cyanotoxicoses have been reported for cattle, sheep, dogs, horses, pigs, coots, ducks, skunks and

mink exposed to water containing cyanobacteria. Animals consuming food in which cyanotoxins may accumulate may also suffer from cyanotoxicoses. Secondary photosensitization may occur in animals exposed to hepatotoxic cyanotoxins. Post-mortem examination may find evidence of cyanobacteria in stomach content, or liver injury. However, in cases of neurotoxin cyanotoxins, no post-mortem lesions are usually found.

## Cyanotoxins in drinking water

Falconer *et al.* (1999) describe how guideline values for cyanotoxins in water were derived. Information from that chapter that can assist in the assessment of risk from such exposure is summarized here. The World Health Organization has established a provisional guideline for human drinking water of 0.001 mg/L of microcystin-LR (total cell-bound and extracellular) (WHO, 2004). Canada has established a cyanobacterial toxin maximum acceptable concentration of 0.0015 mg/L (as microcystin-LR) (Health Canada, 2010). Australia has established a standard of 0.0013 mg/L of total microcystins as microcystin-LR toxicity equivalents (TE) (ANHMRC, 2004). The U.S. EPA has not yet established a standard. Potential carcinogenicity is the effect upon which those standards are based.

Standards for drinking water for animals have not been established.

## Recreational water exposure

Routes of exposure to cyanotoxins for people and animals from recreational waters include: direct contact with the skin (most important for sensitive areas such as the mouth, ears, eyes and throat, and the area covered by bathing suits worn by humans), swallowing and inhalation of water.

Absorption of cyanotoxins after swallowing, contact with nasal mucosa, or inhalation is likely to be important during water-contact activities. Absorption through nasal and pharyngeal mucosa is more likely to occur when activities involve submersion of the head or inhalation of aerosols produced by water spray. Cumulative liver damage may occur by repeated microcystin intake during periods of daily water activities, especially if the activities take place in areas where scums are or have been present.

## Safe practices for drinking water

Draw drinking water from sources that do not harbor cyanobacteria, such as groundwater or surface water not supporting cyanobacterial growth.

**TABLE 99.11** Critical control points for assessing the safety of drinking water

Control point	Remarks
Water source	Groundwater less risky than surface waters. Water sources contaminated by surface runoff pose higher risks
Presence of cyanobacteria; tendency of bloom formation	Bloom formation less likely during periods of high flow or deep vertical mixing, and are more likely during periods of low flow or stagnation. Historical evidence of algal blooms increases risk; blooms may be cyclic. Nutrient input, especially nitrogen and phosphorus, increases risk. Absence of cyanobacteria reduces risk
Cell lysis	Most cyanotoxins are cell-bound and may be removed with the cells. Lysis may occur naturally as the bloom dies, or artificially by treatment, pumping or transport
Water treatment systems	Cyanotoxins may be removed by filtration through activated charcoal or by oxidation, but effectiveness of treatment should be monitored

Adapted from Table 5.1 in Falconer *et al.* (1999).

Cyanobacterial-contaminated water may be used for drinking water if the cyanobacterial cells are removed without lysing them because most cyanotoxins are cell bound. Removal of released cyanotoxins from the water may be possible, but it may also be difficult and expensive. Risk of cyanotoxicosis should be considered high after algae-infested water is treated with copper sulfate. Critical control points for assessing the safety of drinking water are listed in Table 99.11.

All animals should be denied access to bodies of water containing visible discoloration that cannot be attributed to some source other than algae or cyanobacteria. Areas in which scum has formed should be considered very high risk for acute cyanotoxicosis, regardless of the color of the scum. Identification of the microbial components of the scum may help with the risk assessment.

### Safe practices for recreational waters

Surveillance of recreational waters for algal blooms can be effective in minimizing or preventing exposure, but adequate surveillance of such waters for blooms may be difficult. Governmental agencies may not be able to survey all recreational waters within their jurisdiction adequately, so the burden of surveying recreational waters for the presence of blooms may rest with the users of the waters. Once blooms have been detected in public waters, provision of information to the public is usually the responsibility of public authorities.

Algal blooms are usually regarded as more of a nuisance than a toxic hazard. They are often associated

with unpleasant odors and offensive appearances. They may be regarded as normal in areas where they occur frequently or regularly, and their toxic potential may be unknown or disregarded. The presence of any algal bloom, regardless of its color, location, or time of occurrence, should be considered to pose a high risk of cyanotoxicosis and all animals should be denied access to such areas, whether it is in the water or on the shore. The detection of algal scum formation is problematic if routine monitoring is carried out at 1- to 2-week intervals. Scum formation may occur in a matter of hours to days. Daily inspection for scum formation after cyanobacteria are found to be present is recommended.

The availability of ELISA kits for the detection of microcystins makes it easier to monitor recreational waters for the presence of microcystins. Chorus and Cavalieri (2000) do not recommend risk assessment for cyanotoxicoses be based upon chemical analysis for specific cyanotoxins for two reasons: (1) only some of the substances causing health outcomes are known and can be detected; and (2) epidemiological studies indicate some health effects are not due to known cyanotoxins. They recommend that a monitoring program focus surveillance efforts on sites that are likely to present a risk.

### Monitoring strategy for freshwater cyanobacteria

Kuiper-Goodman *et al.* (1999) recommend the following strategy for monitoring freshwater for cyanobacteria, consisting of the three steps listed below. Each will be briefly discussed and serve as a prerequisite for the following step.

- 1 Determine the carrying capacity of the ecosystem for cyanobacteria: Algal and cyanobacterial growth requires nitrogen and phosphorus and the availability of those nutrients limits the numbers of algae and cyanobacteria that can grow in a body of water. Phosphorus is usually the limiting factor of the two and total phosphorus, not ortho-phosphorus, should be measured because algae and cyanobacteria can store sufficient phosphorus to support their growth. If total phosphorus is found to be  $>0.02$  mg/L, then Step 2 monitoring activities should commence. Inspect the water body area to determine if a source or sources of phosphorus or nitrogen input are present, like sewage, fertilization near the water body, or erosion.
- 2 Inspect the site to detect biomass developments: The frequency of monitoring should be no longer than every 2 weeks and should be concentrated in areas in which a biomass most likely will appear, i.e., leeward shorelines. Visual inspections should include the following activities, each serving as its predecessor's prerequisite.



TABLE 99.12 Guidelines for use of recreational waters

Situation	Health risk	Recommended action
Scum is present	High for acute poisoning and short-term health effects (skin irritation, GI illness). Humans are not likely to ingest scum mats or scum-contaminated water, but animals may. Animals may also be exposed when they groom themselves after contact with scum. Young animals or children playing with, in or around scum are at risk	Prevent contact with scum; prohibit water-contact activities
100,000 cyanobacterial cells/mL; 50 µg chlorophyll <i>a</i> /L with dominance of cyanobacteria	Moderate to high for short-term adverse health effects, potential for chronic effects	Watch for scums. Restrict water-contact activities; continue to monitor the situation, post on-site risk advisory signs
20,000 cyanobacterial cells/mL; 10 µg chlorophyll <i>a</i> /L with dominance of cyanobacteria	Low to moderate for short-term irritative or allergenic effects	Post on-site risk advisory signs

Adapted from Falconer *et al.* (1999).

- A Determine water transparency: If the lake bottom cannot be seen at 50cm depth along the shoreline, or if the Secchi transparency is < 1m, then go to Step B.
  - B Determine if greenish discolorations of the water are present along or near the shoreline, indicating the presence of algal or cyanobacterial masses. If so, go to Step C.
  - C Determine if greenish discolorations of the water are present on the surface anywhere on the body of water.
- 3 Quantitatively assess the biomass as the basis for risk assessment when biomass developments occur: Either of two quantitative measurements may be performed: (1) microscopic examination of samples collected from the biomass to identify if cyanobacteria are present, and if so, what kind; (2) quantitate the concentration of chlorophyll *a* present.

Guidelines for the use of recreational waters based upon those measurements are detailed by Falconer *et al.* (1999) and are summarized in Table 99.12.

Falconer *et al.* (1999) do associate microcystin concentrations with numbers of cyanobacteria cells present in water, which may be valuable as an additional risk assessment variable if microcystin-producing cyanobacteria predominate in the biomass. The association is listed in Table 99.13.

Once algal blooms have occurred in a body of water, they should be considered to pose a high risk of cyanobacterial toxicosis until the microorganisms in them have been reliably identified. There are numerous publications on cyanobacteria that describe cyanobacterial blooms as being blue-green in color, but the absence of blue-green coloration in a bloom does not necessarily lessen its toxic hazard. The safest course of action is to deny access by animals to the bloom area.

The single most effective action that animal owners can take to protect their animals from cyanotoxicoses is to

TABLE 99.13 Expected microcystin content of water based upon the amount of microcystin-producing cyanobacteria present

Number of cyanobacterial cells	Expected microcystin concentration
100,000 cyanobacterial cells/mL	Associated with about 20 µg/L of microcystins, if microcystin-producing cyanobacterial predominate
20,000 cyanobacterial cells/mL	Associated with 2–4 µg/L of microcystins, if microcystin-producing cyanobacterial predominate

Falconer *et al.* (1999).

prevent their animals from making any contact with any scum or biomass in the water or on shorelines. It is not usually possible to determine if the scum or biomass is or is not cyanotoxicogenic by visual inspection alone, so any biomass or scum should be considered potentially toxic.

Once an animal has been exposed to a bloom, it is advisable to monitor the animal closely for signs of cyanotoxicoses. Algal scum may be visible on its coat or around its mouth or muzzle and is a good indicator that dermal exposure to cyanobacteria may have occurred and is suggestive that algae may have been ingested. It is not usually possible to determine how much of the scum might have been ingested. It is expected that human swimmers involuntarily swallow 100–200 chlorophyll *a* per mL of water in one session; water skiers and sail-board riders may ingest significantly more.

### CONCLUSIONS

The assessment of the quality of water must be done in the context of its intended use using standards appropriate for the use. The use of quality standards established

for the consumption of water by humans is not as good as use of standards specifically for animals. Quality standards for water consumption by animals have not received as much attention as quality standards for consumption by humans. Nevertheless, several authorities have made recommendations and those standards are likely more appropriate for water quality assessments than are quality standards for humans.

The assessment of risk of adverse health effects upon exposure to water containing cyanotoxins depends upon the use of the water. Standards for drinking water have been published by several authoritative bodies. Such standards are not directly applicable to instances of incidental contact with or the recreational use of bodies of water that may be contaminated with cyanobacteria or their toxins. Measurement of microcystin content for recreational waters may not be comprehensive enough because there are other toxins that can pose a risk that would not be detected by such analytical methods. Assessment is potentially slower as the specimens must be collected, shipped to a laboratory and then tested for the presence of the toxin. Assessment of the presence of algal blooms either by visual criteria or chlorophyll measurement is not dependent upon detection of the presence of an algal toxin, but may restrict water use in the absence of toxin presence. Results based on visual criteria may be faster than those depending upon chemical analysis. Practical advice to animal owners to minimize the risk: deny animal access if any visible presence of an algal bloom either in the water or on its surface or along its bank is detected.

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# Basic concepts of analytical toxicology

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## INTRODUCTION

Veterinary analytical toxicology deals with the chemical analysis of ante-mortem, post-mortem and environmental specimens to assist the veterinary practitioner in the diagnosis of poisoning. The chemical analytical data reported to the veterinarian is of utmost importance. The correct data will lead to correct diagnosis. The erroneous data may lead to disaster; the client may lose large numbers of animals. The erroneous data may also lead to legal ramifications. Thus, correctness of analytical data is of paramount importance for veterinary analytical toxicology. Chemical measurements are based on the principles of analytical chemistry. In practice, the analytical chemist-toxicologist uses analytical methods to achieve the final results. It should be pointed out that by nature, analytical results are variable. The science of trace analysis (analysis at parts per million (ppm) or parts per billion (ppb)) is not as precise as most layman and many scientists view it to be (Rogers, 1986). Analytical methods once devised are, like life forms, subject to evolution. Natural selection as mediated by analytical chemists ensures that only the fittest analytical methods survive. Therefore, an analytical method must be fit for purpose. It should be pointed out that most of the advancements and improvements in the analytical methods have been by making necessary changes and improvements in various steps used in an analytical method. The papers by Sawyer (1988) and Seiber (1988) clearly demonstrate this point for pesticide analysis. The important steps in chemical analytical measurements are (1) decomposition or extraction of the sample, (2) clean up and separation of analyte from bulk impurities (sample matrix), (3) pre-concentration of analyte, (4) detection and measurement

of the analyte, and (5) the generation of the report. The purpose of this chapter is to provide the analytical chemist/toxicologist an overview of the brief history of early and recent developments in chemical measurements, and above all the basis for scientifically sound chemical analytical measurements.

## HISTORY OF CHEMICAL ANALYSIS

### Early history of chemical analysis

Chemical analysis began on the 8th day (McCrone, 1987). Adam, recovering after cooperating with God in creating Eve, felt the first pangs of hunger. He went around and harvested different kinds of colorful berries, and set down for dinner with Eve. Eve rejected some berries due to foul smell (nose as detector). The bitter tasting ones were rejected next (taste as detector), and the delicious ones were consumed. Thus, the first chemical detectors were nose, tongue and eye; *the five senses were used as chemical detectors for a long time.*

The alchemists, in their futile attempts to make gold from base metals, introduced the techniques of filtration, decantation and distillation. Alchemist's zeal, however unsuccessful, leads to a reservoir of observations.

Observations followed observations, and when a large body of knowledge was available, it was named *chemistry*. Those observations that dealt with the production of substances were termed chemical synthesis, and those related to determining what the substances were called were named chemical analysis. The analysis could be carried out in solution as well as in dry state. The

solution methods were termed “wet” methods, and were further divided into volumetric and gravimetric. These methods used sight (visual observation) as detection device. The dry methods also used sight as the detector.

Archimedes, while running naked through the street and shouting *eureka, eureka*, introduced the use of numerical properties for chemical analysis in the concept of specific gravity (density).

The volumetric and gravimetric methods were used for chemical analysis for a long time. These were the principal chemical analytical methods up to the 19th century.

## Recent history of chemical analysis

In recent years, research in analytical chemistry has focused mainly on the development and application of physical and physicochemical analytical methods – instrumental methods of analysis. Instrumental chemical analysis began with Bunsen (Bunsen burner flame) in 1860, who introduced flame emissive spectrometry, and discovered the metals rubidium and cesium. There are a large number of instruments available today for chemical analysis. The most important of these, which have found routine use in veterinary analytical toxicology laboratories, are (1) chromatographic techniques coupled with a large number of detectors, (2) spectrometry and spectrophotometry, both for the visible and ultraviolet range of the light spectrum, (3) atomic absorption spectrophotometry, (4) inductively coupled plasma spectrometry, and (5) mass spectrometry.

Chromatographic methods in general are the most common methods used at the determinative step for the organic compounds. Chromatography is a separation technique, which separates the impurities in the sample solution from analyte. According to International Union of Pure and Applied Chemistry (IUPAC), chromatography is a method used primarily for the separation of the components of a sample in which the components are *distributed* between *two phases*, one of which is stationary while the other moves. The stationary phase may be a *solid*, or a *liquid supported on a solid*, or a *gel*. The stationary phase may be *packed in a column*, *spread as a layer*, or *distributed as a film*, etc. The mobile phase may be gaseous or liquid. Thus there are two movements in chromatographic system, the mobile phase movement – which is usually at a constant rate – and the movement of the components of the sample. The movement of the component depends upon its relative distribution between the stationary phase and the mobile phase. This results in the separation of the components. This separation process coupled with the detection and measuring device completes the chromatographic system. Chromatography can be conducted in gas phase, known as gas chromatography (GC), liquid phase, known as

liquid chromatography (LC), and on a glass or paper coated with solid adsorbent, known as thin layer chromatography (TLC). A variation of LC is known as high-pressure liquid chromatography (HPLC), since high pressure is used to force the solvent through the chromatographic column. Gas chromatographic equipment can be interfaced with large numbers of detectors such as flame ionization (FID), electron capture (ECD), nitrogen phosphorus (NPD), flame photometric (FPD) (responds to phosphorus and sulfur only), Hall electrolytic conductivity (HECD) (responds to halogens only) and mass spectrometer. HPLC can also be coupled with various detectors such as UV/VIS spectrophotometric detector, fluorescence detector, electrochemical detector, refractive index detector and mass spectrometric detector. Chromatographic techniques coupled with a detector are represented by hyphenated abbreviations. For example, a gas chromatograph equipped with FPD is known as GC-FPD. Sometimes this is also represented as GLC-FPD, due to the nature of the stationary phase of the chromatographic column used. Most of the time, a liquid phase is coated on a solid support and packed in a glass column. Recently, silica columns of very small diameter are used in GC, and are known as capillary columns. In these columns, the liquid phase is usually bonded to the inner surface of the silica capillary.

Instrumental methods have surpassed the traditional gravimetric and volumetric analysis in speed and sensitivity.

## TRUENESS (ACCURACY) OF CHEMICAL ANALYTICAL RESULTS

With all the furious activity in the development of various analytical instruments, the question still remains, how good are your results? One can use the most expensive and sophisticated instrument on the market, but still have to prove that the analytical results so obtained are indeed accurate. As mentioned in the introduction, the analytical results by nature are variable; it is more essential to prove the correctness of the results. Let me give a brief history of why this is important.

After the Civil War (1861–1865), American agriculture had been on the march, and farmers had been trying to produce more and more per unit of land. Synthetic fertilizers were used extensively. Various state governments were trying to verify the claims made by the fertilizer manufacturers to protect the consumer (farmer). The problem was that different laboratories got widely different results for the same ingredient in the fertilizers. There were no uniform methods for fertilizer analysis. In order to control this chaos, Mr. J.T. Henderson,



Agriculture Commissioner of Georgia, called a meeting of various interested parties. On May 15, 1884 the meeting took place at the Senate Chambers at the Capitol in Atlanta (Helrich, 1984).

Dr. Charles Shepard, a commercial chemist from Charleston, South Carolina, addressed the meeting and stressed the need for uniform and validated methods for the analysis. He dramatized the effect that nonuniform methods of analysis were having on the monetary value of fertilizers. He told the convention "a 1% difference in the analysis of phosphoric acid made a difference of \$50,000 in the annual production of a 'good-sized factory'." This presentation had a lasting effect on the attendees and it was decided that the group meet again at AAAS, American Association for the Advancement of Science, in Philadelphia, on September 4–10, 1884. The Philadelphia meeting became the first meeting of the "Association of Official Agricultural Chemists," then known as AOAC. This association is now known as AOAC International. Over the past 122 years, AOAC International has developed a process for validating and harmonizing analytical methods. Method validation is the key for determining the performance of analytical methods, and the reliability of the results obtained. AOAC International is committed to analytical excellence and worldwide confidence in analytical results using a defined method validation process.

## ANALYTICAL METHOD VALIDATION

There are several types of validation for analytical methods. AOAC International (Fredrick Ave, Gaithersburg, MD, USA) is the leading organization that conducts methods validation under its auspices. AOAC International conducts the following types of method validation:

- 1 Performance tested method (PTM)
- 2 Peer-verified method (PVM)
- 3 Official methods of analysis (OMA)

AOAC *Official Methods* are referenced in the U.S. Code of Federal Regulations (CFR) and are used worldwide by regulated industry, product testing laboratories and academic institutions. AOAC International is an independent association devoted to promoting methods validation and quality measurements in analytical sciences. It provides full review and validation of approved standard methods of analysis, promotes uniformity and reliability in statements of results, and develops and promotes criteria useful for laboratory accreditation and analyst certification.

### Performance tested methods

There are a large number of rapid test kits available for chemical and biological analysis. In order to verify the claims made by the kit manufacturer about the performance of test kits, AOAC International introduced the concept of PMT. The applicant (kit manufacturer) submits the performance data of the test kit along with the application fee. AOAC International appoints a panel of experts to review the performance data. If the review of performance data is satisfactory, an expert laboratory is selected to check the performance of the test kit. If the performance is as claimed, the test kit is awarded the status of PMT.

### Peer-verified programs

The AOAC® *Peer-Verified Methods*<sup>SM</sup> Program (PVM) is intended to provide a class of tested methods which have not been the subject of a full collaborative study. Through a less intensive process, the program provides a rapid entry point for methods, which are recognized by AOAC at a level of validation for methods not otherwise evaluated. The distinguishing aspect of an AOAC *Peer-Verified Methods*<sup>SM</sup> is that its performance has been checked in at least one other independent laboratory. Eventually, it is expected that most PVMs will undergo full interlaboratory collaborative studies and obtain *Official Methods*<sup>SM</sup> status. An explanation of the PMT, PVT and OMA is available at [www.aoac.org](http://www.aoac.org).

### Single laboratory validation

In order for a method to be validated as PTM, PVM and finally as *Official Method* it must first be validated in a single laboratory. In international trade and regulatory affairs, only validated methods are acceptable. The method need not to be validated under the auspices of AOAC International, but evidence is required that the method is suitable for the intended purpose such that similar results are obtainable from other competent laboratories. In order to gather this information, single laboratory validation (SLV) is essential. Various standards setting organizations such as International Standards Organization (ISO) and AOAC International (AOAC) have established standards for SLV of methods of analysis. AOAC has more than 120 years of experience in collaboratively validating methods of analysis. These standards require that certain performance characteristics (attributes) of an analytical method must meet specified standards. Many analytical methods published in the scientific literature may not meet the criteria specified in the above standards. They usually lack the essential attributes required for the recognized validation process.

The performance characteristics include: the specificity (the ability to distinguish the analyte from other substances); applicability (as the matrices and the concentration range) and reliability. Reliability is the most important characteristic of an analytical method and is expressed in terms of % recovery, repeatability and reproducibility. Repeatability and reproducibility are expressed in terms of relative standard deviation within laboratory and between laboratories, respectively ( $RSD_r$ ,  $RSD_R$ ). Intermediate precision, another term, which is important for SLV, is the intermediate repeatability relative standard deviation ( $RSD_{ri}$ ). It is also very important that the analytical method is fit for its intended purpose. For example, a method intended for the determination of lead (Pb) in drinking water at ppm or ppb level is not fit for the analysis of lead ore (% composition level). If such a method is used for the determination of lead in an ore, the error and the variability will be too large, and the analysis may not serve its purpose, i.e., whether to mine or not to mine a certain location. Thus fitness for purpose is very important.

There are only a few methods that have been validated for use in veterinary analytical toxicology. These methods are listed in the *Official Methods of AOAC International* (AOAC, 2000). An online version of OMA is available as the membership benefit for members of AOAC International. Thus, it is important that any method used in veterinary analytical toxicology is at least validated within the laboratory.

The American Association of Veterinary Laboratory Diagnosticians (AAVLD) is also an accrediting organization for veterinary diagnostic laboratories in the U.S. AAVLD has been emphasizing the use of validated methods in diagnostic laboratories. It should be pointed out that any method from the scientific literature does not become a validated method by simply rewriting it in ISO format. In order to be validated, the laboratory must prove that the method has acceptable performance characteristics of required attributes. If the laboratory hires a new analyst, the analyst should show that he (she) could get results consistent with the performance characteristics of the method. Many federal laboratories such as the Food Safety and Inspection Service (FSIS) and the United States Department of Agriculture (USDA) (FSIS-USDA, 1995) require this.

### AN EXAMPLE OF AN SLV FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF SELENIUM (Se) IN BLOOD AND LIVER

An illustrated example of the SLV for the determination of selenium in liver and whole blood is given here. In our laboratory, there was a need for the determination

of selenium (Se) in fresh liver and whole blood. The literature survey showed a large number of methods for the determination of Se using widely differing techniques such as fluorometry, AA and GLC with electron capture detector (GLC-ECD). Due to the limitation in appropriate instrumentation, and a conversation with another colleague, we decided on the GLC-ECD method. The method of Poole *et al.* (1977) as modified by Ross and Lund (1982) was selected. The method involves the wet digestion of the sample (0.1–1 g) in concentrated nitric acid with magnesium nitrate; the mixture is heated on a hot plate below 100°C until all the liquid evaporates; afterwards, the temperature is raised to remove the brown fumes of nitric acid. The residual nitric acid is removed by placing the sample in a muffle furnace at 500°C for 30 min. After cooling the sample, the ash is dissolved in concentrated hydrochloric acid for 10 min at 100°C. To the solution, 10 ml of 20% urea is added and the mixture is allowed to cool; 4 ml of 1% solution of 1,2-diamino-4-nitrobenzene is added. The solution is transferred to a 50 ml stoppered polyethylene centrifuge tube quantitatively with the aid of 5 ml water. The contents of the tube are mixed, 5 ml of toluene is added to the tube and vigorously shaken for 30 s, and the phases are allowed to separate. About 2 ml layer of toluene is passed through a column of anhydrous sodium sulfate packed in a pasture pipette. The toluene layer is collected in a Pyrex screw cap tube, and 1  $\mu$ l of the toluene (containing Se derivative) is injected in the gas chromatograph fitted with ECD. A Tracor (MT 222) gas chromatograph fitted with a 6 ft glass column with 1/n. internal diameter packed with 1.5% OV-17 and 0.95% OV-225 was used. The oven temperature was 200°C and the detector temperature was 300°C. The carrier gas nitrogen flow was maintained at 60 ml/min. The Se retention time was about 3 min.

### CALIBRATION CURVE: HOW TO MEASURE A QUANTITY OF INTEREST

The modern chemical analytical instruments use physical properties for the measurement of analyte concentration. Thus, analytical instruments have to be optimized and calibrated to detect and report the information about concentration of an unknown. Instruments are calibrated to test the response against a series of known standards of the analyte. The result of this experiment is a plot of response versus concentration, known as calibration curve. Calibration curves are functions of an instrument's response to a range of known concentration levels of reference material. In some cases, the concentration level may be too low to be detected by the particular instrument in use; in other cases, it may be too high and overload the detector. The optimal range

TABLE 100.1 Se calibration curve data

Concentration	Response
$x_i$	$y_i$
0	0
0.1	2.1
0.25	5.8
0.5	10.9
0.75	17
0.99951739	$r$
$b$	$a$
22.5308311	20.049865952

of concentration level for detection is usually narrow. Ideally, this narrow range represents a linear response. The analytical chemist prefers to work in this linear range, since data analysis is comparatively easy in this range. The calibration standards from the reference material should be made with utmost care and accuracy. The concentration of the analyte in the unknown sample is calculated by using a mathematical relationship between concentration and response, known as the regression equation for the calibration curve. The regression equation takes the form response ( $y$ ) = slope of the curve ( $b$ ) +  $y$  intercept.

$$y = bx + a \quad (100.1)$$

We can determine the value of  $b$  and  $a$  from the calibration curve data, and the concentration of analyte in the sample,  $x$ , can be calculated according to Eqn (100.2)

$$x = \frac{y - a}{b} = \frac{\text{Response} - \text{Intercept}}{\text{Slope}} \quad (100.2)$$

The response is read from the instrument, and the slope and intercept are obtained from the calibration curve.

## GOODNESS OF LINEARITY OF CALIBRATION CURVE

The degree of linearity of the calibration curve is determined by the correlation coefficient,  $r$ , the detector response is plotted on the  $y$ -axis and the concentration of the reference standards is plotted on the  $x$ -axis. The value of  $r$  varies from  $-1.0$  to  $+1.0$ . A value of 1 indicates that there is positive linear relationship between the detector response and the standard concentration. The data for a typical calibration curve for Se is shown in Table 100.1. The data in Table 100.1 show a linear relationship, and the calibration curve shows a straight line, shown in Figure 100.1.

The value of  $r$  is calculated on an Excel spreadsheet using the function, =correl(array  $y$ , array  $x$ ). The values

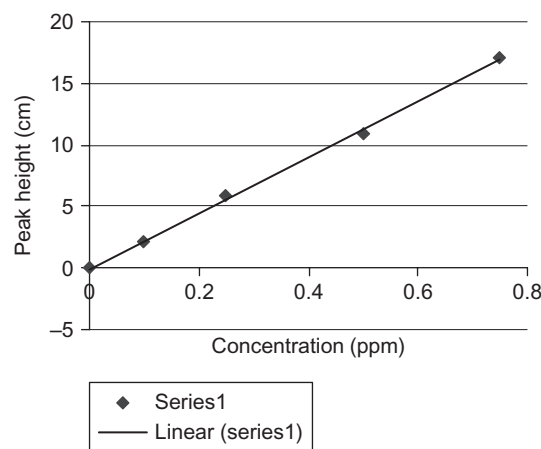


FIGURE 100.1 Se calibration curve.

of the slope  $b$  and intercept  $a$  are calculated by using the function =linest(array  $y$ , array  $x$ ). In order to get both values ( $b$  and  $a$ ), place the data for concentration,  $x$ , and the response,  $y$ , columns as shown in Table 100.1. Run the "linest" function in a column just below, marked  $b$ . Hold the shift and ctrl key and hit enter, both values will appear on the spreadsheet.

The correlation coefficient, slope and the intercept can also be calculated with the aid of Eqns (100.3–100.5) given below (Miller and Miller, 1992):

$$r = \frac{\sum_i \{(x_i - \bar{x})(y_i - \bar{y})\}}{\sqrt{\left[ \sum_i (x_i - \bar{x})^2 \right] \left[ \sum_i (y_i - \bar{y})^2 \right]}} \quad (100.3)$$

$$b = \frac{\sum_i \{(x_i - \bar{x})(y_i - \bar{y})\}}{\sum_i (x_i - \bar{x})^2} \quad (100.4)$$

$$a = \bar{y} - b\bar{x} \quad (100.5)$$

The regression equation for the calibration is as follows:

$$y = 22.53x + (-0.0499)$$

The numbers are referenced in the text. Those without numbers are referred to by other means.

## Recovery and accuracy

The recovery and accuracy was determined with the use of standard reference bovine liver (1577a) from the National Institute of Standards and Technology (NIST), Gaithersburg, MD. The mean value of Se in the NIST standard 1577a was reported to be  $0.71 \pm 0.07$  ppm. Duplicate samples of NIST bovine liver sample were run on 10 different days. A new calibration curve was made

TABLE 100.2 Se recovery data

Replicate	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Mean
1	0.754	0.678	0.614	0.517	0.696	0.769	0.662	0.690	0.560	0.740	0.668
2	0.804	0.753	0.709	0.663	0.691	0.772	0.663	0.619	0.560	0.790	0.7024
Mean											0.6852

TABLE 100.3 Acceptable recovery values for various analyte concentrations

Concentration	Recovery limits (%)
100%	98–101
10%	95–102
1%	92–105
0.1%	90–108
0.01%	85–110
0.001% (10 ppm)	80–115
0.0001% (1 ppm)	75–120
1 ppb	70–125

each day with new Se standards (obtained from Perkin Elmer). The results of Se concentration in NIST bovine liver are shown in Table 100.2.

The mean recovery from NIST 1577a bovine liver is 0.6852 ppm. Thus, recovery % is:

$$\begin{aligned}\% \text{ Recovery} &= (\text{Laboratory mean recovery} / \\ &\quad \text{NIST value}) \times 100 \\ \% \text{ Recovery} &= 0.6852/0.71 = 96.5\%\end{aligned}$$

The % recovery is well within the values recommended in AOAC guidelines (AOAC, 2003) that are shown in Table 100.3.

### Precision

The precision of an analytical method is represented by the standard deviation for the method for replicate measurements. There are two kinds of standard deviations that can be computed for the laboratory method. One is the standard deviation for within day run, i.e., the standard deviation for replicate samples that are analyzed on a given day; the other is the standard deviation for among days analysis. Since the routine samples are analyzed on days when the laboratory receives them, the standard deviation for “among days” applies for the routine samples. The manual calculations for this standard deviation are complex. Fortunately, AOAC International has the software for calculating these standard deviations, which is available from Dr. Joanna Lynch (jl72@cornell.edu), Cornell University, Ithaca, NY. This software is developed for evaluating interlaboratory (collaborative) studies. However, this is also applicable for the determination of within day and among days standard deviation.

This software can also determine the % recovery for the method.

The within day standard deviation ( $s_r$ ), also known as repeatability, is calculated from the software as 0.0480 ppm, and the relative standard deviation,  $RSD_r$ , which is represented by the relationship

$$\%RSD_r = \frac{s_r}{\bar{x}} \times 100 = 7\% \quad (100.6)$$

But our true standard deviation for repeatability is among days standard deviation, thus the true repeatability relative standard deviation is calculated by using  $s_R$  (0.082) (from AOAC software). For replicate measurements made on different days within a laboratory, it is also known as intermediate precision. For the purposes of clarity, this relative standard deviation is represented as %  $RSD_{ri}$ . The true repeatability relative standard deviation (among days) is:

$$\%RSD_{ri} = \frac{s_R}{\bar{x}} \times 100 = 11.97\% \quad (100.7)$$

Although the recovery is within AOAC International guidelines, does it belong to the same population as the NIST bovine liver 1577a? The answer to this question is obvious. The 95% confidence limit for the NIST bovine liver is given in the certificate of analysis as  $0.71 \pm 0.07$  ppm. The laboratory mean recovery of 0.69 ppm (0.6852 rounded to two decimal places) is within the confidence limit as provided by NIST certificate of analysis. Thus, it can be concluded that there is no significant difference between two means. At this point, the only thing to be answered is how good the laboratory relative standard deviation ( $RSD_{ri}$ ) is. This is determined by the use of HorRat values.

### HorRat values

For SLV work, the HorRat value is given by the relationship,

$$HorRat_r = RSD_{ri}/PRSD_R \quad (100.8)$$

where  $RSD_{ri}$  is the relative standard deviation for among days analysis (Eqn 100.8), and  $PRSD_R$  is the predicted relative standard deviation for between laboratories analysis. How do we find  $PRSD_R$ ? It can be predicted based



on the concentration of the analyte (Horwitz *et al.*, 1980; Horwitz, 1982). Based on the data from 100 years' worth of interlaboratory method validation studies conducted under AOAC International auspices, Horwitz determined that relative standard deviation between laboratories and within laboratory is dependent on the analyte concentration. The relationship is expressed by the equation:

$$PRSD_R \% = 2C^{-0.15} \quad (100.9)$$

where  $C$  is expressed as the mass fraction. The above equation can be expressed in spreadsheet notation:  $PRSD_R \% = 2 \times C^{(-0.15)}$ . For example, a concentration of 1 ppm is expressed as  $1.000E-6$  ( $1 \mu\text{g/g}$ ). Thus for an analyte concentration of 1 ppm the calculated:

$$PRSD_R \% = 2 \times (1.000E-07)^{(-0.15)} = 16\% \quad (100.10)$$

Another observation of Horwitz that was confirmed by Thompson and Lowthian (1997) is that the precision of analytical methods at any given concentration does not improve with time, despite the advances in analytical technology. The mean value of selenium in NIST bovine liver is 0.71 ppm, thus, the predicted relative standard deviation for between laboratories is

$$PRSD_R \% = 2 \times (7.1E-7)^{(0.15)} = 16.7\%$$

Thus, the HorRat value for our method for the determination of selenium is:

$$HorRat = 11.97/16.7 = 0.71$$

The accepted HorRat values for single laboratory method validation are from 0.5 to 1.3. Thus, the relative standard deviation for our method is well within the accepted limit. The linearity of the calibration curve, the percent recovery, the relative standard deviation and HorRat value clearly show that the method meets the validation criteria.

## Shewhart chart

The basic concept behind Shewhart's control chart is the distinction between two categories of variation. A process will either display "controlled variation" or it will display "uncontrolled variation." When a process displays controlled variation, its behavior is indiscernible from what might be generated by a "random" or "chance" process. On the other hand, when a process displays uncontrolled variation, then something markedly different has occurred from what would have been expected from a random or chance process, and therefore an "assignable cause" can be attributed to that occurrence. Also, as its

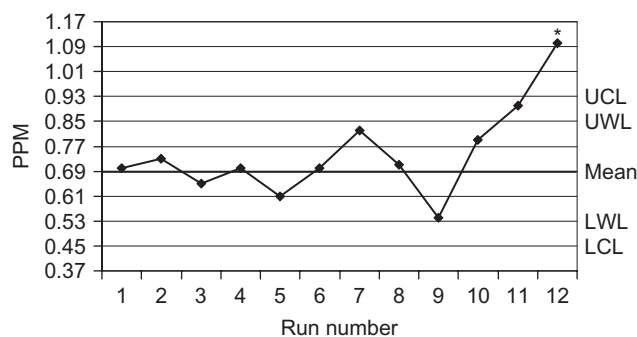


FIGURE 100.2 Shewhart chart – Se assay.

effect has been large enough to notice, it is surely worth the effort to try to identify this assignable cause.

Given this distinction, the control chart is a technique for detecting which type of variation is displayed by a given process. The objective is to give the user a guide for taking appropriate action – to look for assignable causes when the data display uncontrolled variation, and to avoid looking for assignable causes when the data display controlled variation.

Shewhart looked upon the control chart as the voice of the process – one can use the chart to understand how a process is behaving. Therefore, a process is said to be "in control" only when it has demonstrated, by means of a reasonable amount of data, that it is performing consistently and predictably. Moreover, from a practical perspective, such a process is, in essence, performing as consistently as possible. Therefore, any process that is not performing "as consistently as possible" may be said to be "out of control." Thus, the control chart is a procedure for characterizing the behavior of a process.

Figure 100.2 shows the Shewhart control chart for our GC method for selenium determination in NIST bovine liver 1577a. In normal distribution, two standard deviations of the mean consist of 95% of the values, and three standard deviations comprise 99% of all the values, which can result from random variation in the process. Thus, for each run, if we plot the difference from the mean on the  $y$ -axis, and number of runs on the  $x$ -axis, it provides us with a pictorial view of the variation in the process. If our values fall within two standard deviations from the mean, the process is statistically under control. Thus, two standard deviations in either a positive or negative direction form the upper and lower warning limits. If the observation from the run is coming very close to the warning limit line, it means the process is nearing its statistical control limit. If the values go beyond three standard deviations from the mean, it means the process is out of control, and that certain errors other than the random variation are entering the process. At this time, the analysis for routine samples should be stopped, and the reason for the error

investigated. When the problem is solved, only then can routine testing begin. In Figure 100.2, an asterisk marks the point at which the method is out of statistical control.

EXAMPLE OF AN INTERLABORATORY (COLLABORATIVE) STUDY FOR THE VALIDATION OF THE SEMI-QUANTITATIVE METHOD

Any method must be fit for the purpose. Nitrate causes poisoning in ruminants. The nitrate-containing forages are usually responsible for this poisoning. The guidelines for feeding ruminants with nitrate-containing forages are as follows (on dry matter basis) (Guthrie, 1986):

- Forages with nitrate concentrations less than 1000 ppm are considered safe under all conditions.
- Forages with 1000–5000 ppm nitrate should be safe to feed to *nonpregnant animals* under all conditions.
- Forages containing more than 2500 ppm nitrate should be limited to 50% of the total ration for *pregnant and lactating* animals. Erratic and low milk production is expected when nitrate concentration in forages exceeds 2500 ppm.
- Forages containing 5000–10,000 ppm nitrate may be fed safely to *nonpregnant and nonlactating* animals if limited to 50% of the total ration. For *lactating and pregnant animals*, such forage should be limited to 25% of the total ration. Abortions and drop

in milk production should be expected if such forage is greater than 25% of the total ration.

- With forages containing greater than 10,000 ppm nitrate, abortions, acute symptoms and death should be expected. Thus, these forages should not be fed to ruminants.

Results of an intralaboratory study reported by Jain (1993) indicated that nitrate content of forage could be estimated using a test strip. Thus, a collaborative study was conducted to demonstrate the validity of the screening test for nitrate in forages.

Six forage samples were made for the collaborative study (Jain *et al.*, 1999). The nitrate concentration in forage samples ranged from 1000 ppm nitrate to 10,000 ppm nitrate. Five samples were made from field cases submitted to the Athens Veterinary Diagnostic Laboratory. Two of them were mixture of forages. The sixth specimen was also a mixture of forages, and was provided by the Veterinary Diagnostic Center, University of Nebraska, Lincoln. The forage specimens were dried overnight at 60°C in a convection oven, ground using a Wiley Mill to pass through a 2 mm screen, and then thoroughly mixed in a twin shell blender. Samples for each collaborator were prepared by placing 1.0 g of forage in a 50 ml plastic centrifuge tube labeled with a coded number. Five blind replicates of each of the six forage samples were prepared for each collaborator. The samples were labeled with numbers 1–30, and were sent to 20 different laboratories across the U.S.

Table 100.4 shows the results from various laboratories. The laboratory recorded the range number of a given sample. For example, for sample number 21,

TABLE 100.4 Response profiles of laboratory results for each range

	Range 1 samples										Range 2 samples					Range 3 samples					Range 4 samples									
Laboratories	2	11	12	13	16	4	6	10	21	27	8	14	22	28	30	15	17	20	23	29	3	5	7	9	25	1	18	19	24	26
G	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	*2	3	3	3	3	3	4	4	4	4	4	4	*3	4	4
H	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
I <sup>a</sup>	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	*2	*2	*2	3	*4	4	4	*3	4	4	4	4	4	4	4
J	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
K	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
L	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
M <sup>a</sup>	1	1	1	1	1	1	1	1	1	1	*3	*3	2	2	2	*4	*4	*4	3	3	4	4	4	4	4	4	4	4	4	4
N	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
O	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
Q	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	*1	4	4	4	4	4	4	4	4	4
R	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
S	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
T	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	*4	4	4	4	4	4	4	4	4	4	4
U	1	1	1	1	1	1	1	1	*2	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
V	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
W	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	*2	*2	4	4	4	4	4	4	4	4	4	4
X	1	1	1	1	1	1	1	1	1	1	2	2	2	2	*3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
Y	1	1	1	1	1	1	1	1	1	1	2	*3	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4
Z	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4

The \* to the left of the number indicates that the range was incorrectly determined.  
<sup>a</sup>Results of laboratories I and M were deleted from statistical analysis as outliers based on *chi-square* test and inspection, McClure (1990).

which is in range number 1, all the laboratories, except laboratory U, identified the sample correctly in range number 1. The complete results were received from 19 laboratories. The statistical analysis of the data was conducted according to McClure (1990). The published collaborative report does not show how the calculations for statistical parameters were made; it only shows final results. The step-by-step calculations are shown in this chapter for the benefit of the reader.

### Outlier results

First, the results were tested for outlier values, if any, using *chi-square* statistics ( $Q$ ). The null hypothesis is that labs do not have significantly different proportions of correct results. Table 100.5 shows the recast of the data in Table 100.4 for statistical purposes to reflect correct (1) and incorrect (0) results, and is used for computation of the  $Q$  statistic, by Eqn (100.11):

$$Q = \frac{(L-1)(L\sum T_i^2 - (\sum T_i)^2)}{(L\sum S_j - \sum S_j^2)} \quad (100.11)$$

where  $L$  is the number of laboratories reporting the results,  $T_i$  is the number of correct results by the laboratory ( $i$ ) and  $S_j$  is the total number of correct results for sample ( $j$ ). Thus, referring to Table 100.5,

$$Q = \frac{(19-1)(19(16023) - (551)^2)}{19(551) - (10145)}$$

$$Q = \frac{18(836)}{324}$$

$$Q = 46.44$$

This calculated value of chi-square is greater than the critical value of 28.87 for 18 ( $L-1$ ) degrees of freedom. Thus, the null hypothesis is disapproved, and there are significantly different proportions of correct results. By inspection, the laboratories I and M appear to be outliers. After rejecting the outliers the test was repeated, and the calculated value of statistic ( $Q$ ) was found to be 15.775, which is less than the critical value of 26.29 for 16 degrees of freedom (note that the results of laboratories I and M were rejected).

### Sensitivity rate

The sensitivity rate ( $p_+$ ) is the probability that the method will classify a test sample in a "given range" provided that the sample is in that "given range." For example, samples 2, 4, 6, 10, 11, 12, 13, 16, 21 and 27 are range 1 samples; whereas all other samples are in "another range." For

statistical analysis, we are saying that all samples listed under range 1 in Table 100.4 are correctly identified if they are identified as 1 in the table. Any other number in the table for range 1 is considered as misidentification, and is considered a negative value for range 1. The data for the calculations of sensitivity rate and its standard error for various ranges are shown in Table 100.6.

$$(p_+) = \frac{\sum a_i}{\sum m_i} \quad (100.12)$$

where  $\sum a_i$  is the total number of correct results for a given range and  $\sum m_i$  is the total number of results for that range. Thus, the sensitivity for range 1,

$$(p_+) = \frac{169}{170} = 0.994$$

The standard error for sensitivity for each range can be calculated by the formula

$$s.e.(p_+) = \left\{ \frac{(\sum a_i^2 - (\sum a_i)^2/L)}{\sum (\bar{m})^2 L(L-1)} \right\}^{1/2} \quad (100.13)$$

Thus for range 1,

$$s.e.(p_+) = \sqrt{\frac{1681 - 1680.0588}{100 \times 17 \times 16}} = 0.006$$

This means that for range 1, if large numbers of observations were made, 6 out of 1000 observations will misidentify range 1 and 994 observations will correctly identify range 1.

The sensitivity rate and standard error for other ranges can be calculated similarly.

### Specificity rate ( $p_-$ )

The specificity ( $p_-$ ) for the "given range" is the probability that the method will classify the test sample in "any other range" provided the test sample was in "another range." For range 1, all samples that are not in range 1 are in "another range." The value of ( $p_-$ ) can be calculated by Eqn (100.12). For specificity rate calculations,  $m$  is 20 since there are 20 samples in ranges 2, 3 and 4. Thus the total of all  $m$  values is  $20 \times 17$  (340, since there were 17 laboratories). The total of correct values for specificity rate is 339, since only laboratory Q has a range 2, 3 or 4 samples identified as range 1 sample. The data for calculations of specificity rate and its standard error are shown in Table 100.7.

$$\text{Thus specificity rate for range 1 is } (p_-) = \frac{339}{340} = 0.997.$$

[illegible]



TABLE 100.6 Data for calculations of sensitivity rate and its standard error<sup>a</sup>

Laboratories	Range 1		Range 2		Range 3		Range 4	
	$m_j$	$a_i$	$m_j$	$a_i$	$m_j$	$a_i$	$m_j$	$a_i$
G	20	20	25	24	25	24	20	20
G	10	10	5	5	5	4	10	9
H	10	10	5	5	5	5	10	10
J	10	10	5	5	5	5	10	10
K	10	10	5	5	5	5	10	10
L	10	10	5	5	5	5	10	10
N	10	10	5	5	5	5	10	10
O	10	10	5	5	5	5	10	10
Q	10	9	5	5	5	5	10	9
R	10	10	5	5	5	5	10	10
S	10	10	5	5	5	5	10	10
T	10	10	5	5	5	5	10	10
U	10	10	5	5	5	5	10	10
V	10	10	5	5	5	5	10	10
W	10	10	5	5	5	5	10	10
X	10	10	5	4	5	3	10	10
Y	10	10	5	4	5	5	10	10
Z	10	10	5	5	5	5	10	10
Total	170	169	85	83	85	82	170	168
$\Sigma a_i^2$	1681		407		400		1662	
$\Sigma(a_i)^2$	28,561		6889		6724		28,224	
$(\bar{m})^2$	100		25		25		100	

<sup>a</sup> $m_j$  = number of "known" positive samples for a given range/lab;  $a_i$  = number of positive results obtained among "known" positive samples for a given range/lab.

The standard error for specificity rate using Eqn (100.13) is:

$$s.e.(p_-) = \sqrt{\frac{6741 - 6760.059}{400 \times 17 \times 16}} = 0.003$$

### False positive rate ( $p_{f+}$ )

The false positive rate is the probability that the test sample is in "another range" and has been classified in a "given range." The data for the calculations of false positive rate and its standard error is shown in Table 100.8. The equations for the calculations for false positive rate and its standard error are Eqns (100.14 and 100.15), respectively.

$$\text{False positive rate } (p_{f+}) = \frac{\sum a_i}{\sum m_j} \quad (100.14)$$

$$s.e.(p_{f+}) = \sqrt{\frac{(\sum a_i^2 - 2(p_{f+})\sum a_i m_j + (p_{f+})^2 \sum m_j^2)}{L(L-1)(\bar{m})^2}} \quad (100.15)$$

Thus, the false positive rate ( $p_{f+}$ ) for range 1 is  $1/170 = 0.0058823$  rounded to 0.006

$$s.e.(p_{f+}) = \sqrt{\frac{1 - 2(0.0058823) \times 11 + 0.0000346}{17 \times 16 \times 100}} = 0.0058395$$

TABLE 100.7 Data for specificity rate and its standard error<sup>a</sup>

Laboratories	Range 1		Range 2		Range 3		Range 4	
	$m_j$	$a_i$	$m_j$	$a_i$	$m_j$	$a_i$	$m_j$	$a_i$
G	20	20	25	24	25	24	20	20
H	20	20	25	25	25	25	20	20
J	20	20	25	25	25	25	20	20
K	20	20	25	25	25	25	20	20
L	20	20	25	25	25	25	20	20
N	20	20	25	25	25	25	20	20
O	20	20	25	25	25	25	20	20
Q	20	19	25	25	25	25	20	20
R	20	20	25	25	25	25	20	20
S	20	20	25	25	25	25	20	20
T	20	20	25	25	25	25	20	20
U	20	20	25	24	25	25	20	19
V	20	20	25	25	25	25	20	20
W	20	20	25	23	25	25	20	20
X	20	20	25	25	25	24	20	20
Y	20	20	25	25	25	24	20	20
Z	20	20	25	25	25	25	20	20
Total	340	339	425	421	425	422	340	339
$\Sigma a_i^2$	6761		10,431		10,478		6761	
$\Sigma(a_i)^2$	114,921		177,241		178,084		114,921	
$(\bar{m})^2$	400		625		625		400	

<sup>a</sup> $m_j$  = number of "known" positive samples for a given range/lab;  $a_i$  = number of negative results obtained among "known" negative samples for a given range/lab.

### False negative rate ( $p_{f-}$ )

The false negative rate is the probability that a test sample is in a "given range" and the sample has been classified in "another range." The data for the calculations of false negative rate and its standard error are shown in Table 100.9. Thus, the false negative rate ( $p_{f-}$ ) and its standard error for various ranges can be calculated from the Eqns (100.14 and 100.15), respectively.

False negative rate ( $p_{f-}$ ) for range 1 =  $1/340 = 0.0029411 = 0.003$  and its standard error is

$$s.e.(p_{f-}) = \sqrt{\frac{1 - 2 \times (0.003) \times 21 + 0.0612}{17 \times 16 \times 400}} = 0.0029 = 0.003$$

### Performance parameters for the method

Various performance parameters are shown in Table 100.10.

## LABORATORY QUALITY MANAGEMENT

The integrity of a laboratory product depends on the quality system that is followed by the laboratory. Just what is quality and why is it so important? Quality system has a different meaning in different laboratories but

TABLE 100.8 Data for calculations of false positive rates and their standard error

Laboratories	Range 1				Range 2				Range 3				Range 4			
	$m_j$	$m_j^2$	$a_i$	$a_i m_j$	$m_j$	$m_j^2$	$a_i$	$a_i m_j$	$m_j$	$m_j^2$	$a_i$	$a_i m_j$	$m_j$	$m_j^2$	$a_i$	$a_i m_j$
G	10	100	0	0	6	36	1	6	5	25	1	5	9	81	0	0
H	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
J	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
K	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
L	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
N	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
O	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
P	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
Q	11	121	1	11	5	25	0	0	5	25	0	0	10	100	0	0
R	10	100	0	0	5	25	0	0	5	25	0	0	9	81	0	0
S	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
T	10	100	0	0	5	25	0	0	4	16	0	0	10	100	0	0
U	9	81	0	0	6	36	1	6	5	25	0	0	11	121	1	11
V	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
W	10	100	0	0	7	49	2	14	3	9	0	0	10	100	0	0
X	10	100	0	0	4	16	0	0	5	25	0	0	10	100	0	0
Y	10	100	0	0	4	16	0	0	5	25	0	0	10	100	0	0
Z	10	100	0	0	5	25	0	0	5	25	0	0	10	100	0	0
$\Sigma m_j$	170				87				82				169			
$\Sigma m_j^2$		1702				453				400				1683		
$\Sigma a_i$			1				4				1				1	
$\Sigma a_i m_j$				11				26				5				169
$\Sigma a_i^2$			1				6				1				1	
$(\bar{m})^2$	100				26.19				23.27				98.52			

<sup>a</sup> $m_j$  = the number of positive readings for a given range among positive and negative test samples/laboratory;  $a_i$  = the number of positive readings for a given range among known negative samples/laboratory.

TABLE 100.9 Data for calculations for false negative rate and its standard error<sup>a</sup>

Laboratories	Range 1				Range 2				Range 3				Range 4			
	$m_j$	$m_j^2$	$a_i$	$a_i m_j$	$m_j$	$m_j^2$	$a_i$	$a_i m_j$	$m_j$	$m_j^2$	$a_i$	$a_i m_j$	$m_j$	$m_j^2$	$a_i$	$a_i m_j$
G																
H	20	400	0	0	24	576	0	0	26	676	1	26	21	441	1	21
J	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
K	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
L	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
N	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
O	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
P	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
Q	19	361	0	0	25	625	0	0	25	625	0	0	21	441	1	21
R	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
S	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
T	20	400	0	0	25	625	0	0	26	676	1	26	19	361	0	0
U	21	441	1	21	24	576	0	0	25	625	0	0	20	400	0	0
V	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
W	20	400	0	0	25	625	0	0	27	729	2	54	20	400	0	0
X	20	400	0	0	26	676	1	26	24	576	0	0	20	400	0	0
Y	20	400	0	0	26	676	1	26	25	625	0	0	20	400	0	0
Z	20	400	0	0	25	625	0	0	25	625	0	0	20	400	0	0
$\Sigma m_j$	340				425				428				341			
$\Sigma m_j^2$		6802				10,629				10,731				6843		
$\Sigma a_i$			1				2				4				2	
$\Sigma a_i m_j$				21				52				106				42
$\Sigma a_i^2$			1				2				6				2	
$(\bar{m})^2$	400				625				633.9				402			

<sup>a</sup> $m_j$  = number of negative readings among both "known" positive and negative samples lab;  $a_i$  = number of negative readings among "known" positive test samples/lab.

TABLE 100.10 Performance parameters for various ranges

Parameter	Range 1	Range 2	Range 3	Range 4
Sensitivity rate ( $p_+$ ) <sup>a</sup>	0.994	0.977	0.965	0.988
Standard error of ( $p_+$ )	0.006	0.016	0.026	0.008
Specificity rate ( $p_-$ ) <sup>b</sup>	0.997	0.991	0.993	0.997
Standard error of ( $p_-$ )	0.003	0.005	0.004	0.003
False positive rate ( $p_{f+}$ ) <sup>c</sup>	0.006	0.046	0.012	0.006
Standard error of ( $p_{f+}$ )	0.006	0.025	0.012	0.006
False negative rate ( $p_{f-}$ ) <sup>d</sup>	0.003	0.005	0.007	0.006
Standard error of ( $p_{f-}$ )	0.003	0.003	0.006	0.004

<sup>a</sup>The sensitivity rate ( $p_+$ ) is the probability that the method will classify a test sample in a "given range" provided the sample is in that "given range."

<sup>b</sup>The specificity rate ( $p_-$ ) is the probability that the method will classify the test sample in "another range" provided the test sample is in "another range."

<sup>c</sup>The false positive rate ( $p_{f+}$ ) is the probability that the test sample is in "another range" and has been classified in a "given range."

<sup>d</sup>The false negative rate ( $p_{f-}$ ) is the probability that a test sample is in given range, and the sample has been classified in "another range."

they share the same key elements to ensure the quality and integrity of the product. A quality system has elements that are of a quality assurance (QA) nature and others that are of a quality control (QC) nature. The quality system is also referred as QA/QC. Some laboratories work under the standards of a certain organization such as ISO, AOAC, or AAVLD, whereas some laboratories develop their own quality system customized to the needs of the laboratory (Tebbett, 2003).

### Quality assurance

ISO standard 9000:2000 defines QA as part of the quality management focused on providing the confidence that quality requirements will be fulfilled. All QA programs have similar elements: they are designed to ensure that (1) the sample is not contaminated, (2) is handled by personnel who are trained for that specific job and are using equipment that is properly calibrated and procedures that are validated, and (3) that everything is documented and that records can be accessed when needed (Tebbett, 2003). Good quality produces good results. It gives the laboratory an edge over competitors and makes the customers happy because they can rely on the integrity of the results produced. Poor quality wastes time, supplies and money (Archer, 2005). The accuracy of the result is questionable, which casts a shadow on everything that the laboratory produces.

### Quality control

Quality control refers to the steps taken during the analytical process to ensure that the process produces reliable measurements, data that are fit for the purpose.

## CONCLUSIONS

"Prove it!" What would be your response to that challenge to the data generated by your investigation or services? Can you prove the accuracy of your data? Yes, you can prove the integrity and accuracy of your results if the validated analytical method was used in conjunction with reference materials traceable to a recognized source, and you have at hand the validation data. Just saying that we use validated methods in our laboratory is not enough. The analysis of reference materials traceable to sources recognized by measurement bodies with client samples is essential to provide defensible data of such quality that is useful to the client. Even the validated methods must be repeated in the laboratory to confirm that the laboratory is able to get the performance parameters as reported in the validated method. The record keeping of the validation of performance parameters is part of the quality management plan. In order to ascertain that the method is performing under statistical control, a control sample should be run with each batch of samples. The value of the control sample gives the indication of the performance of the method on a given day. Analyzing control samples with each batch of samples is also part of the quality management program. Each laboratory must have a sound quality management (QA/QC) program.

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# Sample submission for toxicological analysis

Beverly S. Arnold

## INTRODUCTION

Is this animal poisoned? This question arises in almost every veterinary practice. An accurate diagnosis is the single-most important factor in dealing with poisoned animals. The offending agent may be known or unknown. If the offending agent is known, specific treatment and prevention can be initiated. Often the toxic agent is unknown. In this instance, efforts are limited to supportive care and symptomatic therapeutic measures.

Chemical analysis is very important in diagnosing poisoning. This is usually done at veterinary diagnostic laboratories. In the U.S. there are 41 AAVLD accredited veterinary diagnostic laboratories. A list of accredited and allied laboratories is available at [www.aavld.org](http://www.aavld.org). Most of these laboratories are partially funded by the respective states, thus, the services of veterinary diagnostic laboratories are available at a reasonable price to the client. Familiarity with the chemical analysis services provided by the diagnostic laboratory in your area and its fee structure are extremely important in the effective use of the services provided by the laboratory.

The objective of this chapter is to provide guidelines for proper sample submission to diagnostic laboratories. Each diagnostic laboratory has a submission form, which should be completed in full to get the most efficient service for chemical analysis. All of the diagnostic laboratories require complete history, clinical signs and post-mortem findings. This information greatly aids the laboratory personnel to decide on the best course of chemical analysis.

## REQUIRED INFORMATION FOR SAMPLE SUBMISSION

### History

A detailed history of the circumstances associated with poisoning is the first step for a successful diagnosis. The investigation of a poisoning case is as much detective work as it is veterinary medicine and chemical analysis. Three detectives are partners in this endeavor – veterinarian, client and the analytical laboratory. The veterinarian must be ready to gather as many pieces of the puzzle together as possible. [Fitzgerald \(2001\)](#) described in detail various elements of a good history. [Osweiler \*et al.\* \(1985\)](#) and [Osweiler \(1996\)](#) provided a checklist for a good history.

### Clinical signs

Clinical signs are very important in reaching the diagnosis of a poisoning. Clinical signs can narrow the search for toxic agents. Ask the client for the sequence of clinical symptoms. Describe clinical signs in detail. It is not enough to say that the patient was observed with central nervous system (CNS) signs. It is essential to provide the complete sequence of events as to how the patient acted.

### Post-mortem findings

If the animal has died, send the body to a veterinary diagnostic laboratory. A pathologist qualified in

necropsy techniques will conduct the necropsy and collect the appropriate samples for histological as well as for toxicological testing. If it is poisoning then why worry about necropsy and histological examination? Simply test for toxicants and it should give us a diagnosis. Unfortunately, it is not that simple. There are thousands of chemicals that can cause poisoning in animals. *There is no simple test for all the poisons.* Some poisons produce extensive lesions, others only slight tissue alterations, and some produce no lesions at all. Based on this information the pathologist can provide assistance in the selection of toxicants to test for.

### Chemical analysis

Chemical evidence is often an indispensable aid in diagnosing toxicological problems. Used properly, chemical analysis can provide the most important evidence of poisoning. Analytical data should be used in conjunction with history, clinical signs, necropsy findings and histological examination. The importance of clinical signs, post-mortem findings and chemical analysis has been described in detail in previous reports (Osweiler *et al.*, 1985; Poppenga and Braselton, 1990; Gale, 2000, 2001, 2004; Puschner and Gale, 2001; Volmer and Meerdink, 2002).

## SAMPLE COLLECTION AND SUBMISSION FOR TOXICOLOGICAL ANALYSIS

A list of various types of samples which can be useful in the investigation of poisoning cases is given in Table 101.1.

Each sample should be stored in a separate container, and the container should be labeled with an indelible ink with animal and tissue identification. Place the sample container in a zip-lock bag, squeeze the air out of the bag, and seal and label it. *Do not add anything to the samples for chemical analysis: no antiseptic, no preservative, no fixative, unless otherwise noted (as for whole blood).* These instructions apply to all the samples which are collected. Lorgue *et al.* (1996) provides a detailed account for sample collection and dispatch to the laboratory with particular reference to United Kingdom.

### Whole blood

Whole blood should be collected in royal blue top (for trace elements) Vacutainer tubes with anticoagulant. At least 10ml of whole blood should be collected. If more

than one test is required, collect 20ml blood. Make sure the tube is properly sealed. Refrigerate the sample. *Do not freeze whole blood sample.*

### Serum

The blood for serum samples should be collected in royal blue top Vacutainer tubes (plain) which contain *no additives or anticoagulants*. After the clot forms, in usually less than 30min, the serum should be separated promptly, and transferred to a clean plastic tube and frozen.

### Urine

Urine should be collected in a screw cap plastic bottle (4oz.) and frozen.

### Liver biopsy specimen

Collect approximately 100–200mg of liver sample and immediately freeze; some laboratories may not be able to provide results for some metals due to small sample size. Contact the laboratory before proceeding with biopsy.

### Milk

Collect in a clean jar and refrigerate.

### Feces

Place feces in heavy duty aluminum foil, wrap it completely, place in zip-lock bag and freeze.

### Vomit or gastric aspirate

Place vomitus in a screw cap plastic bottle and freeze. In case of gastric aspirate make sure to submit first aspirate.

### Hair

Hair samples should be collected using clean scissors or clippers. Do not contaminate with other materials such as blood on gloves. Place the hair sample in a zip-lock bag. Keep it at room temperature.

### Brain, liver, kidney, fat and other tissue(s) with lesions

Collect appropriate amount of the particular sample (Table 101.1), place the sample on heavy duty aluminum

TABLE 101.1 Samples for toxicology testing

Sample	Amount	Tests for which sample can be used	Comments
<i>Ante-mortem samples</i>			
Blood	10–20 ml	Heavy metals, trace elements, anticoagulants, antifreeze, cholinesterase, cyanide, chlorinated hydrocarbon pesticides	Refrigerate sample. Use anticoagulant such as EDTA or heparin. Use royal blue tubes with anticoagulant
Serum	10–20 ml	Some metals like Cu, Zn, and nitrate/nitrite, ammonia, drugs (particularly sulfa drugs)	Separate clot from the serum. Refrigerate or freeze (if it will take long during shipping). Amount required for single test will be much less. Check with the testing laboratory. If Zn assay is desired use royal blue tubes without any additive or anticoagulant
Urine	50–100 ml	Alkaloids, some metals, antibiotics, cantharidin, drugs, sulfa drugs, oxalates and paraquat or diquat	Freeze. Especially useful for paraquat or diquat
Feces	250 g	Drugs, various poisons excreted mainly in bile	Freeze. Indicator of recent exposure
Biopsy specimen: liver		Metals	Freeze
Milk	250 ml	Antibiotics, chlorinated insecticides, polychlorinated biphenyls	
Vomitus or gastric	All available	Various poisons. Heavy metals, anticoagulants, antifreeze, pesticides (all chlorinated hydrocarbons organophosphates, carbamates)	Freeze. Indicator of recent exposure. In case of gastric aspirate make sure to submit first aspirate
Hair	5–10 g	Pesticides, and some metals such as As and Se	Indicator of recent exposure. Chronic accumulation of metals
<i>Post-mortem samples*</i>			
Brain	Half	Chlorinated pesticides, pyrethrins, cholinesterase, and metals like, Pb, Na, Hg	Brain should be separated by midline sagittal section. Collect frontal cortex for cholinesterase determination. Freeze
Fat	250 g	Chlorinated pesticides and dioxins	
Kidney	100 g or all available	Metals like As and Pb. Phenolic compounds, oxalates	Freeze
Liver	100 g or all available	Chlorinated pesticides, alkaloids, anticoagulants, aflatoxin M <sub>1</sub> and metals	Freeze
Ocular fluid	All available	Nitrate/nitrite electrolytes	Freeze
Rumen content	500 g	Various poisons. Heavy metals, pesticides (all chlorinated hydrocarbons organophosphates and carbamates)	Freeze. Sample should be collected from several locations in the rumen. Rumen content is not a good sample for nitrate/nitrite evaluation
Stomach content	500 g or all available	Various poisons. Heavy metals, anticoagulants, antifreeze, pesticides (all chlorinated hydrocarbons organophosphates and carbamates)	Freeze
<i>Environmental samples</i>			
Baits	All available	Various poisons. Heavy metals, anticoagulants, antifreeze, pesticides (all chlorinated hydrocarbons organophosphates and carbamates), anticoagulants	Any other information which is available about bait should be communicated to the testing laboratory
Feeds	2 kg	Various poisons. Heavy metals, pesticides (all chlorinated hydrocarbons organophosphates and carbamates), ionophores	Multiple representative samples should be taken from various places from the stock and mixed thoroughly for a composite sample or retained as individual samples to detect variability in the feed or both. Feed samples are required for ionophores screening
Forage (pasture)	2–5 kg	Various poisons. Usually used for evaluation of nitrate/nitrite	Samples should be taken from multiple locations in pasture and mixed thoroughly for composite sample. Avoid contamination from soil. Refrigerate sample
Forage (hay)	2–5 kg	Various poisons	Samples should be taken from multiple locations using a forage core sampler from baled hay or stacks and mixed thoroughly for composite sample
Forage (silage)	2–5 kg	Various poisons	Freeze
Mushrooms	Whole 100 g	Identification cyclic peptide hepatotoxin	Keep cool and dry in paper bag
Plants	Entire plant	Identification	Press between sheets of paper
Soil	1 kg	Various poisons	Samples should be taken from multiple locations using a soil core sampler and mixed thoroughly for composite sample
Water	1 l	Nitrate/nitrite, pesticides and metals	Use clean glass container. Refrigerate sample

foil, fold the corners of aluminum foil to seal the sample and place the foil with the sample in the appropriate size zip-lock bag. Handle one specimen at a time and avoid cross contamination. Freeze the tissue samples; fat may be refrigerated. Special attention is needed for brain samples. Brain should be separated by midline sagittal section. Collect the frontal cortex for cholinesterase assay (Gupta, 2004) and freeze the sample until analysis. It should be pointed out that caudate nucleus should not be used for cholinesterase assay, since frontal cortex is a better indicator of cholinesterase status (Gupta, 2004).

### Ocular fluid

Collect ocular fluid in a small plastic tube and freeze.

### Ingesta (rumen content and stomach content)

Carefully examine the contents of rumen or stomach for extraneous material. Make sure that the extraneous material is sampled for submission. Collect appropriate amount of the particular sample (Table 101.1), place the sample on heavy duty aluminum foil, fold the corners of aluminum foil to seal the sample and place the foil with the sample in the appropriate size zip-lock bag. Rumen contents should be collected from several locations in the rumen and mixed thoroughly. Rumen content is *not* a good sample for nitrate/nitrite evaluation.

### Baits

Collect all possible bait samples, wrap in heavy duty aluminum foil and place in a zip-lock bag. Gather all possible information regarding the bait, and forward the information to the testing laboratory along with the sample.

### Feedstuffs

Feedstuffs should be collected from several different locations in bin, crib, or silo. Many mycotoxins, for example, are found in isolated pockets of the feed and therefore could be missed if one sample is obtained. It is preferable to sample some feed from the troughs or bunks. If the ration is mixed on the premises, it is advisable to collect at least one pound each of the various components in the total ration. Samples of dry ration can be submitted in plastic bags, and the bag labeled with the identification of the sample.

### Forage (pasture)

Samples should be taken from multiple locations in pasture and mixed thoroughly for composite sample. Avoid contamination from soil. Refrigerate the sample.

### Forage (hay)

Ideally, hay samples should be taken from multiple locations using a forage core sampler from baled hay or stacks and mixed thoroughly for composite sample. Additional attention should be paid when sampling hay for nitrate/nitrite assay. Nitrate is water soluble. When a bale of hay is exposed to rain, nitrate leaches from the top of the bale and percolates through the bale. In this instance, it is preferable to sample hay in the area where the water line has stopped. This area usually has very high nitrate concentration. Since this sample is wet, freeze it promptly. Make a notation on the history sheet if the animals were observed to eat hay from this area. In addition, the samples from other parts of the bale should also be submitted.

### Forage (silage)

Silage samples should be taken from multiple locations and frozen promptly.

### Mushrooms

Samples of mushrooms are collected for two purposes: identification of mushroom species and chemical identification of cyclic peptide hepatotoxin. For identification, collect a whole mushroom, and keep it cool and dry in a paper bag. For the presence of cyclic peptide hepatotoxin, collect about 100g sample and freeze.

### Plants

Plant samples are collected for identification of the plant as well as the identification of plant toxins. It is desirable that the whole plant is collected intact, with roots and any flowers or buds for identification. Plants can be pressed between sheets of newspaper and sent for identification. Plants for chemical analysis should be wrapped in aluminum foil, placed in a labeled plastic bag and frozen.

### Soil

Samples of soil for chemical analysis should be taken from multiple locations using a soil core sampler and mixed thoroughly for composite sample.



## Water

Collect samples of water in clean glass containers.

## Samples for histological examination

In addition to samples for chemical analysis, samples of tissues fixed in formalin should also be submitted for histological examination. The importance of histological examination of tissue samples has already been discussed in an earlier section of this chapter.

## Samples for nutritional elemental analysis

Whole blood and serum are analyzed for this purpose. For elemental analysis, whole blood should be collected in royal blue top Vacutainer tubes. For serum samples, the blood should be collected in royal blue top tubes (plain), which contain *no additives or anticoagulants*. After the clot forms, in usually less than 30 min, the serum should be separated promptly and transferred to a clean plastic tube and frozen. The manuscript by [Hancock et al. \(1988\)](#) provides excellent information about the number of animals to be sampled for laboratory analysis for herd health management. It contains information tables for sample size required to estimate prevalence or attribute with a given confidence limit.

## Submission forms

All of the veterinary diagnostic laboratories have submission forms. Cases are not accepted without submission forms. Complete the submission form as thoroughly as possible. Include all the information as discussed in earlier sections. Remember that there are three detectives in solving the poisoning problem – the client, the practitioner and the analytical toxicologist at the diagnostic laboratory. It has been our experience that an accurate, fully completed submission form greatly helps laboratory colleagues to take the most appropriate analytical approach.

## SHIPPING REGULATIONS FOR DIAGNOSTIC SPECIMENS

Most countries have regulations for shipping diagnostic specimens. In the United States the rules are published in the “Code of Federal Regulations” (CFR) Part 49, sections 100–185. It is available on the internet at <http://ecfr.gpoaccess.gov/>.

## Packing diagnostic specimens for transport: summary

- You as the shipper – not the transport company – are responsible for the shipment until the package reaches the consignee.
- The diagnostic specimens should be packaged in triple packing, consisting of primary, secondary and the outer packing.
- *Primary packaging*: Primary receptacle(s) must be water tight, e.g., screw cap seal with Para film or adhesive tape or similar. Multiple primary receptacles must be wrapped individually to prevent breakage.
- *Secondary packaging*: Use enough absorbent material in the secondary container to absorb the entire contents of all primary receptacles in case of leakage or damage. Secondary packaging must meet IATA packaging requirements for diagnostic specimens including the 1.2 m (3.9 ft) drop test procedure.
- Secondary packaging must be watertight. Follow the packaging manufacturer’s or other authorized party’s packing instructions included with the secondary packaging. Secondary packaging must be at least 100 mm (4 in.) in the smallest overall external dimension. Must be large enough for shipping documents, e.g., air waybill.
- *Outer packaging*: The outer packaging should be rigid and of adequate strength for the intended use.
- Each package and the air waybill are labeled differently depending on whether the contents are infectious or non-infectious animal specimens. Infectious substances are considered dangerous or hazardous materials. National and international regulations require that the person involved in the packaging and shipping of hazardous materials be trained and certified in the proper handling of these materials.
- An itemized list of contents must be enclosed between the secondary packaging and the outer packaging. Place in a sealed plastic bag to protect from moisture.
- A shipper’s declaration for dangerous goods is *not* required if the sample was taken directly from the patient, it does not contain an infectious substance and the container is labeled “Exempt Animal Specimens.”
- *Fines and penalties*: According to 49 CFR Sec. 171.1 (c) “Any person who knowingly violates a requirement of the Federal hazardous material transportation law...Is liable for a civil penalty of not more than...\$27,500...and not less than \$250 for each violation...and shall be fined under Title 18, United States code, or imprisoned for not more than 5 years, or both.”

## Dispatch of samples

- Place one or two ice packs (as used for camping) in a polystyrene box. Place all frozen sample packets over

ice packs. If whole blood is shipped together, make sure that some insulating material is between the frozen samples and blood. Place the polystyrene box in a rigid box.

- Write the address of the receiving laboratory legibly on the center of the outside box. On the upper left-hand corner write the sender's address.
- The submission form should be attached to the outside of the secondary packing and sealed in a plastic sleeve.
- The parcel should be clearly marked "diagnostic specimen" on the outside packing, and sent either by U.S. parcel post, UPS, FedEx, or any other courier. Check with the courier for their special requirements.

## CONCLUSIONS

Great care should be taken during the collection and shipping of diagnostic specimens for chemical analysis. Remember that laboratory results are as good as the submitted specimen. Follow transportation rules carefully, or any violation will involve a fine. Diagnostic laboratories are not the enforcers of transportation rules. There was an incident of a shipping foul-up by a practitioner. The courier called the laboratory to say that they had a package for delivery to the laboratory. However, courier staff were unwilling to handle the package, since it was shaking and making funny noises. The laboratory was asked to retrieve the package right away. The senior member of the laboratory went to pick up the package, opened it and found a live piglet in the container. Fortunately, the laboratory was informed the day before by the practitioner that he was sending a euthanized piglet for examination. Apparently, the piglet was not given enough euthanasia solution. This incident happened about 10 or more years ago. Imagine what would happen today if a package like this arrived at the courier!

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# Toxicoproteomics in diagnostic toxicology

Christina R. Wilson and Stephen B. Hooser

## INTRODUCTION

During the past decade, advances in genomics research have resulted in the completion of entire genome sequences for multiple species. This plethora of genetic information, compiled in genome sequence databases, has been used to characterize changes in gene expression in response to external stimuli, such as toxicants (toxicogenomics). Because genomic data reflects changes in gene expression at the mRNA level, DNA sequence databases can be queried to predict what proteins may be present in the cell. However, this approach is problematic because there is a poor correlation between mRNA levels and protein concentrations in cells (Gygi *et al.*, 1999a; Anderson and Seilhamer, 2005). Additionally, the biological activity of proteins is controlled by post-translational protein modifications or protein–protein interactions, both of which cannot be predicted by the genomic data. Therefore, characterizing the entire protein complement expressed by genes (proteome) in response to external stimuli serves as a better indicator of the response of an organism, tissue, or cell to toxic insult. This has given rise to the “proteomic” approach to toxicological evaluation. Toxicoproteomics can encompass global analysis of all of the proteins expressed, called “shotgun proteomics,” or can entail analyzing a subset of proteins of interest, often termed “targeted proteomics.” Regardless of the approach, proteomics-based research has created an analytical challenge, requiring sophisticated, high-throughput analytical techniques and complex computer algorithms.

## ANALYTICAL TOOLS FOR PROTEOMIC ANALYSES

### The proteome and sample complexity

The initial step in proteome analysis typically involves the separation of intact proteins or peptides generated from proteolytic digestion of protein mixtures. Analysis of these heterogeneous components has proven to be an analytical challenge due to the large dynamic range of proteins and the microheterogeneity of protein expression in biological samples. For instance, serum albumin concentrations can range from 35 to  $50 \times 10^9$  pg/mL and proteins of lower abundance, such as interleukin 6, can range in concentration from 0 to 5 pg/mL (Anderson and Anderson, 2002). This is also complicated by protein heterogeneity due to polymorphisms, alternative mRNA splicing, or variations in post-translational modifications. When analyzing global proteolytic digests of proteins (i.e., at the peptide level), the sample complexity is compounded further. Proteolytic digests of the serum proteome, which could contain as much as 20,000 proteins, can potentially result in 200,000 to 600,000 peptides (Anderson and Anderson, 2002; Issaq *et al.*, 2005). Therefore, one can appreciate the analytical challenges encountered when conducting proteomics research in complex biological systems. Attempts to overcome these drawbacks have imposed improvements in sample preparation and separation methodologies. Multidimensional levels of separation can be achieved when these techniques are combined affording increased resolution, sensitivity and accuracy of detection.

## Two-dimensional gel electrophoresis

Since its inception in 1975 (O'Farrell, 1975), two-dimensional gel electrophoresis (2-DGE) has become one of the most common platforms for separating and profiling complex protein mixtures. This technique is two dimensional in that there are two levels of protein separation. The first dimension uses immobilized pH gradient-isoelectric focusing, which separates proteins based on differences in net charge (Görg *et al.*, 2000). In the second dimension, proteins are resolved by electrophoresis and separated based on molecular weight. Visualization of resolved protein spots is achieved using universal stains such as Coomassie blue, Coomassie brilliant blue (SeePico™), silver, negative-reversible zinc, fluorescent, or radioisotope labeling dyes (Görg *et al.*, 2000; Lopez, *et al.*, 2000; Ong and Pandey, 2001; Kuramitsu *et al.*, 2010). If a particular subproteome is targeted for detection, more specific gel stains can be used. For example, detection of target proteins can be accomplished using stains containing antibodies for those proteins of interest or post-translationally modified proteins can be visualized using specialized stains for phosphorylated and glycosylated proteins (Görg *et al.*, 2004; Vlahou and Fountoulakis, 2005; Otani *et al.*, 2011).

After staining, the gel is digitized and protein concentrations quantitated using sophisticated 2-DGE image analysis software. Comparative analysis between control samples and samples of diagnostic interest can be accomplished using these image analyzers to superimpose the digitized data from multiple gel runs. However, due to the laborious nature of this procedure and the lack of gel reproducibility, comparative analysis is often difficult. In order to circumvent these limitations, fluorescent dyes, known as Cy dyes, have been developed permitting simultaneous analysis of two samples on one gel (Ünlü *et al.*, 1997; Hamdan and Righetti, 2002). This technique, called two-dimensional differential in-gel electrophoresis (2-D DIGE), involves labeling each sample with one of two different Cy dye fluorophores, mixing the two samples together, and analyzing them using 2-DGE and fluorescent gel imaging.

While the gel-staining techniques used to visualize and quantitate proteins may vary, in all instances protein identification is commonly accomplished using tandem mass spectrometry (MS/MS). Excised protein spots are digested into peptides using proteolytic enzymes and subjected, offline, to MS/MS analysis. The peptide mass fingerprints generated and peptide sequence data are then compared to theoretical peptide masses in protein or genome sequence databases using specialized bioinformatics algorithms.

Despite the fact that improvements in 2-DGE technology have enabled high resolution of separated proteins and enhanced protein identification, some intrinsic

problems remain. Limited throughput capabilities, inability to measure low and high molecular mass proteins, inter-gel variability and inefficient detection of basic and hydrophobic proteins are still inherent limitations of this proteomics platform. Regardless of these limitations, 2-DGE is still a powerful tool for the separation of intact proteins and is the most widely used technique in comparative toxicoproteomic analyses.

## High performance liquid chromatography

Attempts to improve protein separations have warranted the development of gel-free systems for large-scale analyses. Chromatographic strategies used to fractionate proteins and peptides have proven to be a successful alternative to 2-DGE. The chromatographic techniques commonly employed in proteomics research include affinity chromatography, capillary electrophoresis (CE), hydrophobic interaction chromatography, hydrophilic interaction chromatography, ion exchange chromatography (IEX), reversed-phase chromatography (RPC) and size exclusion chromatography (SEC) (Goheen and Gibbins, 2000; Levison, 2003; Goetz, *et al.*, 2004; Mahn and Asenjo, 2005; Mirzaei and Regnier, 2005; Babu *et al.*, 2006; Mondal and Gupta, 2006; Wilson *et al.*, 2008; Di Palma *et al.*, 2011). These modes of chromatography fractionate proteins and peptides based on their adsorption and desorption on stationary phase supports through mobile phase manipulation. On the protein level, they are commonly used to pre-fractionate samples in order to simplify complexity prior to analysis by 2-DGE or high performance liquid chromatography (HPLC). Fractionation of proteins using these methods can also be accomplished online using high throughput HPLC techniques. While this approach has proven to be a successful initial step in protein purification, HPLC fractionation of intact proteins is uncommon in proteomics.

HPLC is, however, the most widely used analytical tool for separating proteolytic digests of complex protein mixtures. In this approach, all of the proteins in the sample are digested into peptides using a proteolytic enzyme. After digestion, the peptides are separated using HPLC with UV detection. When interfaced with a mass spectrometer, the amino acid sequences of these peptides can be determined and their respective parent proteins identified using bioinformatics software.

Large-scale analysis of proteolytic digests can compromise the resolving power of HPLC when only one chromatographic fractionation technique is used (Davis and Giddings, 1985a,b). Therefore, orthogonal approaches using multidimensional chromatographic separations are preferred, affording greater peptide resolution and accuracy of detection. The most widely used high throughput separation technique is the coupling of IEX



and RPC with MS instrumentation. Other examples of multidimensional HPLC/MS techniques can also include RPC/CE chromatography, affinity chromatography/RP chromatography, SEC/IEX/RPC and SEC/RPC/CE (Issaq *et al.*, 2005; Zhang *et al.*, 2010a).

HPLC has also been used for quantitative analysis of protein expression. Relative quantitation of proteins by HPLC is based on the theory that the concentration of the parent protein can be determined by the relative peak areas of UV-detected peptides observed from that protein (Chelius and Bondarenko, 2002). Another HPLC-based approach for relative protein quantitation involves the use of internal protein standards (Bondarenko *et al.*, 2002). In this technique, biological samples are spiked with an internal standard protein prior to enzymatic digestion of the proteins into peptides. The chromatographic peaks observed can be normalized to the peak area of the internal standard and the relative concentrations of the proteins are inferred.

Unlike 2-DGE/MS tools, multidimensional HPLC/MS is amenable to high throughput analyses and has the ability to resolve peptide mixtures regardless of molecular mass or hydrophobicity. This is important because resolving peptide mixtures by 2-DGE is impractical due to their narrow isoelectric points and molecular weight ranges and because 2-DGE tools cannot be directly interfaced with MS instrumentation. Even though 2-DGE is superior when separating intact proteins, methods based on pairing multidimensional HPLC and MS technology are becoming more refined and continue to advance proteomics research.

## Protein and peptide microarrays

Protein and peptide microarray technologies offer a complimentary approach to traditional separation methodologies and mass spectrometry. This technology incorporates the use of a variety of high throughput microarray platforms to probe protein function, abundance and to globally analyze protein expression in biological systems. The platforms commonly used in proteomics include proteome, antibody, reverse-phase protein and lectin microarrays (Lina *et al.*, 2011).

Protein function microarrays use immobilized capture ligands to screen for protein–drug, protein–enzyme, protein–protein interactions (Kawahashi *et al.*, 2003; Nielsen *et al.*, 2003; Zhu *et al.*, 2003; Cretich *et al.*, 2006). Analytical microarrays, such as those used in surface-enhanced laser desorption (SELDI)/TOF MS, use retention chromatography for protein profiling and detection (Merchant and Weinberger, 2000; Cretich *et al.*, 2006). This technique is capable of on-chip sample fractionation utilizing various chromatographic surface chemistries

and can probe for chemical properties in proteins such as phosphorylation, glycosylation, hydrophobicity, or anionic–cationic properties.

Microarrays require immobilization of a capture ligand or chromatographic media onto a planar solid support, often resulting in thousands of spots per slide (MacBeath and Schreiber, 2000; Kumble, 2003). Samples of interest are added to the microarray and the entire array is scanned to detect proteins or peptides that interact with the ligand or chromatographic media.

Detection strategies for protein or peptide microarrays entail either label-free or labeled probe methods (Espina *et al.*, 2004; Cretich *et al.*, 2006). The label-free strategy is a direct detection method that includes MS, surface plasmon resonance, or atomic force microscopy. SELDI microarray chips commonly use MS-based detection of proteins or peptides. Labeled probe methods of detection include utilizing chromagens (similar to ELISA protocols), chemiluminescence, fluorescence, or radioactive decay-based detection techniques.

Microarray technology is progressively becoming a versatile platform for its potential use in diagnostic toxicology. Although efforts to standardize array analyses have been challenging, microarrays make it possible to simultaneously screen thousands of samples and profile large numbers of proteins from biological samples.

## Mass spectrometry

Recent developments in hybrid mass spectrometers have revolutionized the ability to analyze proteins and peptides, providing high throughput automation combined with high sensitivity and resolving power. In general, mass spectrometers have three components, an ion source, a mass analyzer and a detector. The ion source is the component in which protein and peptide ions are produced; the mass analyzer separates or resolves these ions based on their mass-to-charge ( $m/z$ ); and the detector detects the selected ions from the mass analyzer. One stage mass analysis is commonly used to measure the molecular weights of intact proteins or peptides. However, in order to obtain peptide sequence information, hybrid mass spectrometers must be employed. MS/MS, or tandem mass spectrometry, detects intact peptide ions in the first mass analyzer. Once selected, the peptide ion enters the collision cell where it is fragmented into secondary ions by collision-induced dissociation (CID). These ions are then separated in the second mass analyzer, their  $m/z$  ratios detected and MS/MS spectra are generated. Bioinformatics database tools use the MS/MS data to generate peptide amino acid sequence information and compare them with theoretical sequences in protein or genome databases to identify proteins.

There are two major types of hybrid mass analyzers used in proteomics research, matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF/TOF) instruments and electrospray ionization/tandem MS instruments (ESI/MS/MS) (Karas and Hillenkamp, 1988; Fenn *et al.*, 1989; Hillenkamp *et al.*, 1991). The MS technology chosen depends on the type of MS data that needs to be generated from toxicoproteomic experiments. For example, MALDI-TOF/TOF instruments are fast, robust mass analyzers, have a large dynamic range and do not require labor-intensive sample preparation. MALDI-TOF/TOF instruments have high resolution, making them the instruments of choice when using mass spectrometry for quantitative proteomic analyses. ESI/MS/MS instruments, including those containing hybrid combinations of quadrupole, 3-D ion trap, linear ion trap, Orbitrap, TOF and Fourier transform-ion cyclotron resonance mass analyzers (FTICR) have approximately four times less peak capacity than MALDI-TOF/TOF; however, they have higher mass accuracy affording more accurate protein identification (Hopfgartner *et al.*, 2004; Hu *et al.*, 2005; O'Connor *et al.*, 2006; Yates *et al.*, 2006; Merchant, 2010). Additionally, they are capable of analyzing low molecular weight peptides and can be directly interfaced with HPLC instrumentation.

Innovative approaches to advance protein identification strategies have spurred the development of new MS technologies. Improvements in ion activation using electron capture dissociation (ECD) or infrared multiphoton dissociation technologies have been shown to yield more extensive peptide sequence coverage when compared to CID, resulting in significant improvements in protein identification (Wysocki, *et al.*, 2005; Bakhtiar and Guan, 2006). Although once thought to be impossible, characterization of intact, large proteins can be accomplished using ECD combined with FTICR (Sze *et al.*, 2003; Zhang *et al.*, 2010b). Developments in MALDI imaging mass spectrometry have paved the way to simultaneously map peptides and proteins by direct MS analysis of thin tissue sections, providing a means to correlate and monitor changes in protein patterns associated with regions of the tissue that are histologically significant (Mange *et al.*, 2009; Stauber *et al.*, 2010). Other significant technological advances in large biomolecule ionization and data analysis have enabled the development of miniaturized, portable mass spectrometers capable of direct analysis of complex biological samples (Laughlin *et al.*, 2005; Cooks *et al.*, 2006).

In addition to being essential for protein identification, MS technology is being used for quantitative proteomic profiling. Through the use of stable isotope-coded mass tags, differential quantitation of changes in peptides from control and experimental samples is possible. Quantitation of changes in global protein expression

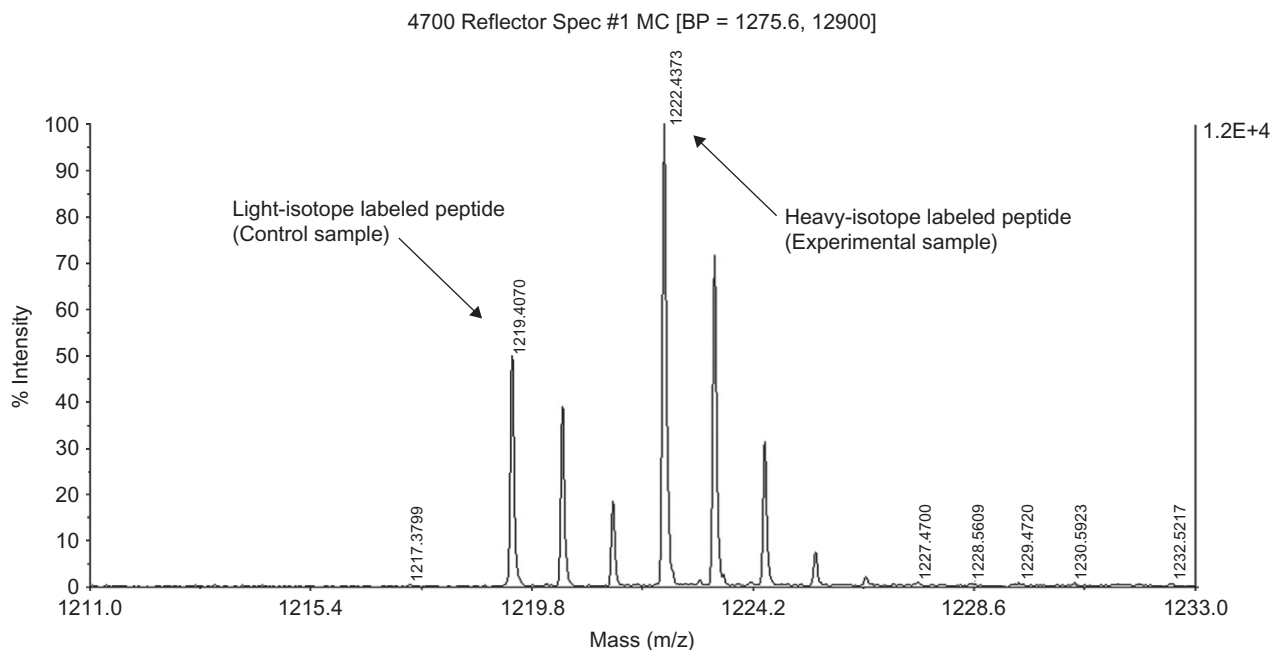
TABLE 102.1 Isotope-coded reagents used for chemical labeling of peptides

Reagent	Labeling technique	Reference
ALICE	Thiol modification of cysteines	Qui <i>et al.</i> (2002)
AQUA	Synthetic internal standard peptide	Gerber <i>et al.</i> (2003)
ICAT	Iodoacetylation of cysteine	Gygi <i>et al.</i> (1999b)
iTRAQ™	Modification of primary amines	Zieske (2006)
GIST	Acylation of primary amines	Ji <i>et al.</i> (2000)
MCAT	Guanidination of C-terminal lysine	Cagney and Emili (2002)
QUEST	Amidation of N-terminal lysine	Beardsley and Reilly (2003)

involves proteolytic or chemical labeling of peptides with isotope-coded mass tags prior to separation by HPLC. These labeling reagents are chemically identical; however, one label contains light isotope atoms and the other heavy isotope atoms. During proteolysis, enzymatic cleavage results in the incorporation of oxygen at the peptide carboxy-terminus. Exploiting this reaction, <sup>18</sup>O (heavy) and <sup>16</sup>O (light) isotopic oxygen labels can be used to differentially label two samples (Stewart *et al.*, 2001; Ye *et al.*, 2010). Other examples of isotope-coded reagents and their labeling strategies are listed in Table 102.1. In isotope tagging, peptides from a control sample are labeled with the light isotope and the peptides from the treated sample are labeled with the heavy isotope. After labeling, both samples are mixed together and fractionated using HPLC. When subjected to MS analysis, the mass spectrometer can distinguish between the two isotope-labeled peptide samples because a predictable mass difference will be observed between the control and experimental peptides. MS-based quantitation is then achieved by calculating the difference between the ion intensities of the light-labeled control peptide and the heavy-labeled experimental peptide samples (Figure 102.1). From this data, differential displays of peptides that increase or decrease in response to a stimulus can be generated. These differential displays are commonly used to generate protein expression profiles or protein signature patterns that can be used in comparative toxicoproteomic investigations.

## Bioinformatics tools

Protein identification is accomplished by using computer search algorithms that correlate MS and MS/MS data with predicted amino acid sequences contained in protein or genome sequence databases. Even though several types of MS and MS/MS search engines have been created, databases that are used more frequently include MASCOT, SEQUEST, Spectrum Mill, X!Tandem and Protein Prospector (Eng *et al.*, 1994; Clauser *et al.*, 1995;



**FIGURE 102.1** MALDI-TOF MS spectrum of GIST isotopically labeled peptides from control and experimental serum samples. Comparison of the relative ion intensities between the two peptides indicate that the peptide present in the experimental sample (at 1222.43  $m/z$ ) was present at a higher concentration when compared to the control sample (at 1219.40  $m/z$ ).

Perkins *et al.*, 1999; Robertson and Beavis, 2004; Kapp *et al.*, 2005). Database resources have also been created to probe for post-translational modifications on proteins and peptides. These database search engines include UniCarb-DB, for glycomics, and PHOSIDA 201, for common post-translational modifications (Gnad *et al.*, 2011; Hayes *et al.*, 2011). Tox-Prot, a searchable toxin protein database, has been created that can be queried for most known animal protein toxins (Jungo and Bairoch, 2005).

Even though the computer algorithms designed for most bioinformatics databases are slightly different, their general approach to protein identification is similar. Database search engines compare the experimental precursor  $m/z$  ions from each MS/MS scan with hypothetical peptide  $m/z$  values from the database. Hypothetical peptide masses from the database that correspond with the experimental mass values are assigned probability scores. The proteins recognized with the highest scores are indicative of the best probable protein match to the experimental MS/MS data. Some bioinformatics tools assign  $p$ -values to the correlation scores, providing an additional means for evaluating credibility of protein matches.

Integrating MS technology with bioinformatics tools has become an indispensable tool in proteomics research. However, due to the overwhelming amounts of MS and MS/MS data generated from typical proteomics experiments, creating bioinformatics tools that adequately identify and characterize the data has been a tremendous

challenge. Credible protein identification is reliant on successful interpretation of MS and MS/MS data. Unfortunately, data interpretation is often complicated by ion suppression, atypical MS/MS peptide fragmentation patterns, ill-defined universal standards for evaluating credible database matches and inability to define a single protein from one peptide spectrum (Kearney and Thibault, 2003). Additionally, variants of MS instrumentation are developing rapidly and the computer algorithms necessary to correlate the data from these next generation mass spectrometers with information contained in protein or genome sequence databases will continue to be a challenge.

## PROTEOMICS APPLICATIONS IN DIAGNOSTIC TOXICOLOGY

The current objectives of toxicoproteomics in diagnostic toxicology is to define molecular mechanisms of toxicity, screen for drug toxicities and elucidate biomarkers or signature protein profiles in order to more accurately assess, predict and diagnose toxicities (Kennedy, 2002; Guerreiro *et al.*, 2003; Wetmore and Merrick, 2004). For decades, laboratories have relied on individual protein markers for assessing toxicity. However, some of these single biomarkers can be non-specific and reflect protein

leakage from tissues, as opposed to the direct effects of toxicants on the tissues alone (Plebani, 2005). Toxicities in biological systems are multifactorial and complex, emphasizing identification of multiple biomarkers for accurately diagnosing and classifying toxicity. This makes proteomics research in toxicologic evaluation appealing because these technologies are capable of globally profiling multiple proteins. Hence, the potential to better define molecular signatures of toxicity for clinical and diagnostic toxicology is becoming increasingly possible. Several proteomic applications have been applied to gain a better understanding of target organ toxicities, mechanisms of toxicity and biomarkers of exposure for a variety of toxicants.

### Aquatic toxicology and pesticides

In veterinary toxicology, pesticide use and potential non-target toxicity in aquatic organisms is always a concern. In order to gain a better understanding of the mechanisms of toxicity, changes in protein expression have been assessed in fish exposed to single or multiple pesticides. For example, proteomic analyses of proteins in brain responsive to methyl parathion in zebrafish (*Danio rerio*) have been investigated. In this study, it was found that six proteins, which were predominantly cytoplasmic proteins, significantly changed with methyl parathion exposure (Huang *et al.*, 2011). The proteins most affected by methyl parathion are known to be involved in catalysis, binding, cell structure and metabolic regulation. In another study in brains from male, flathead minnows (*Pimephales promelas*), protein expression was compared in fish exposed to permethrin, terbufos, or a binary mixture of both pesticides (Biales *et al.*, 2011). In this study, 24 proteins changed with exposure to these pesticides when compared to controls. Some of the proteins identified are known to be associated with the cytoskeleton, glycolysis, hypoxia and the ubiquitin–proteasome system. The investigators also compared protein expression patterns in treated fish versus control fish. Interestingly, the permethrin protein expression pattern was distinguishable from that in the controls, enabling them to discriminate between the two treatment groups with 87.5% accuracy. When comparing the proteins identified in the aforementioned studies, the protein dihydropyrimidase-like 3 was down-regulated in zebrafish exposed to methyl parathion; however, this protein was up-regulated in the flathead minnows exposed to permethrin.

### Hepatotoxicity

Monitoring liver function is crucial in toxicologic evaluation. Hence, it is no surprise that toxicoproteomic

experiments have been conducted to gain a better understanding of proteins that change with hepatotoxicity. For example, toxicoproteomics has been used to identify metabolic pathways affected by drug-induced cirrhosis in rat models. In these studies, thioacetamide and ethanol have both been shown to down-regulate proteins involved in oxidative stress and fatty acid  $\beta$ -oxidation pathways during early phases of cirrhosis (Low *et al.*, 2004; Venkatraman *et al.*, 2004). Ivermectin hepatotoxicity has been investigated in gilthead sea bream fish (*Sparus aurata*). Of the proteins that significantly changed, 30 proteins decreased and six proteins increased with exposure to ivermectin (Varo *et al.*, 2010). The major hepatic proteins that changed were apoA-1 (lipid metabolism),  $\beta$ -globin and ATP synthase subunit beta (oxidative stress response and regulation of energy).

### Toxins

Valuable information regarding toxicological evaluation of venom (“venomics”) has also been made possible through toxicoproteomic investigations. These investigations have been used to identify protein toxin components in venom, assess geographic venom variability and evaluate antivenin efficacy. In one study conducted in venom from the Asian forest scorpion (*Heterometrus petersii*), 10 families of venom peptides and proteins were identified. The proteins identified in the venom were potassium channel toxins, antimicrobial and cytolytic peptides, calcium channel toxins, La1-like peptides, phospholipase A2, serum proteases, acid phosphatases and diuretic peptides (Ma *et al.*, 2010). Comparative proteomic analysis of pit viper venom (*Bothrops atrox*) has been used to show geographic variation in venom phenotypes and to assess antivenin reactivity. The protein toxin phenotype in pit vipers from Colombia and Venezuela were recognized to be typical of the adult phenotype; whereas the toxin phenotype from Brazil resembled that of the juvenile snake (Calvette *et al.*, 2011). The significance of this finding was relevant to the treatment of envenomation by this type of snake, as it was shown that the antivenins used for pit viper snake bites in these regions were more immunoreactive against the venom in the pit vipers with toxin phenotypes similar to juvenile snakes (Brazil region).

Other natural toxins examined using proteomics include mycotoxins. It is known that drought stress and high temperatures provide opportunistic conditions for fungal growth in corn and other susceptible crops (Guo *et al.*, 2008). In an attempt to gain a better understanding of what makes certain corn crops more susceptible to aflatoxin contamination from *Aspergillus flavus*, a proteomic comparison of corn kernel proteins in resistant and susceptible crops was evaluated. Using 2-DGE and



ESI/MS/MS, proteins were identified in corn kernels resistant to *Aspergillus flavus* that included proteins related to stress-tolerance (aldose reductase and glyoxalase 1 protein) and antifungal proteins T1 and PR10 (Chen *et al.*, 2002).

## CONCLUSIONS

Recognition of the diagnostic accuracy of proteomics data and standardization of methodologies remain a challenge to proteomics researchers. Despite these concerns, toxicoproteomics has the potential to better define molecular mechanisms of toxicity and elucidate biomarkers or signature protein profiles for clinical and diagnostic toxicology. This technology promises to establish new guidelines for preventive and predictive toxicity, ultimately revolutionizing and redefining our future understanding of toxicology.

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# Microscopic analysis of toxic substances in feeds and ingesta

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## INTRODUCTION

Feed microscopy is the scientific discipline and technology for the identification and evaluation of feed ingredients and formula feeds. Basically there are three essential components to a successful microscopic identification. These include an aid to the eye, a trained mind and a collection of reference standards. The feed microscopist uses both the stereomicroscope and the compound microscope to identify unknown particles by comparisons to known reference standards. The experience and training of the microscopist determine the efficacy and speed of the identification. A microscopic analysis can be applied to any type or size of particles but commonly observations range from the macroscopic level for the initial gross examinations to the stereoscopic level for centimeter- to millimeter-sized opaque to translucent particles. Translucent and transparent fine particles are examined with the compound microscope to confirm the particle identity at the cellular and sub-cellular levels. In addition, due to the ability of the human mind to sort and classify a wide range of different materials rapidly, the feed microscopist can accomplish a subjective analysis of a feed ingredient in just a few minutes that might take a chemist several hours to accomplish.

It is important to understand from the beginning that feed microscopy is subjective and visual in nature. It is not an objective technology that generates hard numbers. It is designed to identify the feed resources and their inherent quality visually but not to determine the quantity of the individual nutrients within those feed

resources. Therein is the real essential value of feed microscopy to the feed manufacturer, quality assurance laboratory, veterinary toxicologist or anyone concerned about feed ingredient quality and its effects on the consuming animal. Feed microscopy is the entire other half of the quality assurance picture that is often discounted because it involves an opinion. It is, however, a powerful technology that plays an important role in the evaluation of any feed or toxicant analysis and an understanding of what microscopy can do needs to be introduced into every general veterinary and toxicology curriculum.

Microscopy is critical to the toxicological examination of feeds because it is the most rapid and practical way to define problems and focus on potential solutions. Although feed microscopy has not generally been a part of veterinary school curricula, feed microscopists have been part of the diagnostic picture for many years. Veterinary diagnostic laboratories and poison control centers, often located in universities or in a state chemist's office, have utilized feed microscopists as part of their rapid response teams. Unfortunately the number of feed microscopists that are trained and available for veterinary diagnostic work today has declined dramatically to the point that integrating a feed microscopist into the initial toxicological examination is now very difficult. Because the microscopist can sort through more seemingly unrelated but potentially pertinent information about a feed than any other type of analyst and handle more complex situations in feeds than a chemist using standard methods, we need to reinsert a feed microscopist back into the modern veterinary fast response diagnostic team.



For example, there are absolutely no rapid chemical assays to locate blister beetles or their fragments in alfalfa samples and the analysis of the cantharidin toxicant would take several hours. In contrast, a feed microscopist should be able to locate specific evidence of the beetles in a few minutes without any sample preparation. The knowledge of how and where to look for potential problems in feeds is also a critical part of defining a problem. Although it is more specific to look for the toxin itself, the low concentrations of toxins in feeds make them difficult to detect. It is often more efficacious to find an indicator of the toxin and take appropriate remedial action than spend the time in sample preparation and detailed confirmatory analysis.

## THE SEARCH FOR CLUES

Feed ingredients and finished feeds come in a bewildering array of sources, particle sizes, mixtures and forms. Added to that the other natural materials that are fed, or that animals consume, and the task of identifying toxicological problems appear totally impossible. Certainly that would be the case if we did not have an organized way of identifying and classifying all the potential ingredients in a feed. Basically the majority of feed ingredients and formula feeds are byproducts from the food industry. Because they are materials processed out of foods, they are often wet and require drying to be safely stored prior to use. They may also be relatively dry but hygroscopic and difficult to store. They could also be dry but susceptible to oxidation or rancidity due to having been removed from their cellular compartmentalization in nature. For these and numerous other reasons, they are all potential problems for the toxicologist if they are not handled or stored properly. Nevertheless, the clues to identifying which ingredient(s) or feeds are or are not a problem in any situation is right in front of the toxicologist and available to be interpreted. The clues for feed quality are inherent in the visible appearance of each ingredient.

## BACKGROUND: PRINCIPLES OF FEED MICROSCOPY

Feed microscopy is founded on the most basic descriptive aspects of biology and the wide diversity of cellular structures found in natural organisms that are genetically controlled and that do not change from year to year. It is also based on the fact that many of these unique tissues, cellular traits and sub-cellular

characteristics are not greatly affected by the moisture, heat, pressure and shear commonly used to manufacture formula feeds. Therefore, not only are these cellular characteristics inherently good identifiers of individual feed ingredients and mixtures, they are equally good for finished formula feed identifications because they maintain their characteristics after they have been intimately mixed, conditioned, pelleted, extruded, or otherwise manufactured into finished feeds. Consequently, feed microscopists can trust these characteristics to make accurate identifications of ingredients and confirm them to the cellular and sometimes the sub-cellular levels. If a micro-chemical or micro-biochemical test is applied to the sample, the feed microscopist can confirm ingredients to the molecular level and watch the reaction in real time in the cells. In addition, they can evaluate feed ingredients, finished formula feeds and, in special instances, the ingesta and fecal wastes from feeds and forages. The latter is a specialized part of the feed microscopy field but one that is particularly important for the toxicologist. In all these situations, the feed microscopist is very much like a medical examiner or a veterinary toxicologist who uses the unique visual characteristics of organ or tissue morphology, red or white blood cell characteristics or sub-cellular tissue sections to diagnose diseases or other pertinent problems.

Feed microscopy is, in concept, a simple subjective science. It is sometimes further defined as being both art and science due to the need to have steady hands and to develop very good manipulative skills under the microscope. Therefore, if we build on the basic three essentials and add a few hand tools to the well-trained mind, the microscopes and the numerous reference standards mentioned above, we can produce a complete analytical system.

Feed microscopy, due to its conceptual simplicity, is a rapid and relatively accurate technology for identifying materials and problems for which no other direct tests or scientific instruments exist. It is also a relatively rapid way to identify animal problems and it can become an essential complement to objective laboratory analyses. For example, a feed microscopist can identify the residues in a feed bunker that had been rejected by cattle, the contents of dog vomit to identify a feed source or a problem, or perhaps locate evidence of mold growth or mites hidden in a pelleted feed. Subsequently the microscopist can, after locating evidence of a potential feed problem, advise the analytical or toxicology laboratory staff to select appropriate methods to confirm, negate or quantify the extent of the problem. Thus, the feed microscopist is very much like a diagnostician who examines an animal and, based on multiple observed criteria, concludes subjectively that certain definitive laboratory tests should be run to confirm the diagnosis and potential cure.

The feed microscopist, like the diagnostician and toxicologist, must be well versed in the significance of tiny observed differences from the expected norms. Toxicants are not visible at light microscope resolution and feed microscopists are not trained to evaluate tissue samples, organ morphology or animal behavior. However, the particles and structures associated with toxicity such as yew leaves, mold growth or moldy feed, a blister beetle wing fragment, castor bean seed coat fragments and many others can be detected and identified using common feed microscopy methods. These are the keys to both the identification of potential feed problems and the selection of suitable chemical testing plus the evidence to correlate with the findings of the diagnostician.

Because there are often no specific tests for the small differences that must be detected to ascertain a particular problem, the feed microscopist uses all possible senses to evaluate the feeds. For example, although there is no single instrument that can identify an unknown sample as fish meal or ground corn without a lot of work, a feed microscopist can make those qualitative determinations in a matter of minutes. Thus, organoleptic observations, color of particles, breakage lines, presence of caramelized and charred particles and many other seemingly insignificant details may offer clues to the quality of a feedstuff or help to explain a suspected toxicant situation. An objective laboratory test would be necessary to confirm or deny the actual presence of the suspected toxicant.

Another very important facet of feed microscopy is to detect natural contaminants that are similar to the feed ingredients and particularly for adulterants that are deliberately selected to deceive and not be detected by standard methods. An example of a classical adulterant would be urea used in an ingredient for a monogastric animal ration. The ingredient could command a premium price due to the apparent superior protein content. It would not be detected by the feed manufacturer testing for crude protein. Unless non-protein nitrogen testing was routinely run to detect this type of protein spiking or if a microscopist spotted the urea particles and ordered a non-protein nitrogen test, this adulteration would escape detection.

It should have become obvious by this point that a feed microscopist familiar with the structural characteristics of plants, animals and microbial materials and trained in the proper use of the microscope or other aid to the eye can not only solve certain difficult to solve types of problems such as those of feed quality, but can often aid the chemist or other laboratory analyst to select appropriate tests to solve increasingly more complex problems that we often encounter today. Feed microscopy is particularly well suited for solving complex multifaceted contaminant, adulterant and toxicological problems and should be part of any rapid response team.

## THE APPLICATIONS OF FEED MICROSCOPY

Although the concepts of feed microscopy are quite simple, there are numerous ways to achieve accurate results. The more techniques and methods the microscopist masters, the easier it will be to handle the wide range of sample matrices and problem samples that will be encountered. Some techniques require essentially no sample preparation whereas others might require flotation, sieving or other separations to assist the analyst. There are no absolutes in feed microscopy as is the case with most subjective examinations. Therefore, it is essential that the microscopist have a full understanding of the available techniques and methods so that the most appropriate tests are applied and the most scientifically accurate information can be established from the available evidence in any sample. If the sample size is limited, then certain tests might have to be run before others so that the reagents of the first test will not confound the results of subsequent tests. Thus, the feed microscopist should be well versed in chemistry and biochemistry to be a key member of a rapid response team.

The trained mind of the feed microscopist may also be thought of as a computer with a massive hard drive and an extreme logic program to sort through the hundreds of facts and "what if" possibilities. That would be true also of any diagnostician dealing with complex situations. One way to understand and appreciate the capabilities of feed microscopy and some of the variables that should be considered in the sorting process to the most applicable methods and techniques for a particular sample is to consider what can be seen at various magnifications.

Feed microscopy is often defined by the level of magnification used. The initial examination would be analogous to a gross morphological description to establish the general direction of each later stage of an analysis. The stereomicroscope or dissecting microscope is the basic workhorse for most feed microscopists just as it is for the veterinary diagnostician or toxicologist. It would be used to aid the eye in a general review of the surfaces of pelleted feed, for example, although a lighted magnifier could be used as well. An examination of a feed ingredient or finished feed generally requires at least 7× magnification to see particles ground through a typical 2mm hammermill screen. On site, field work can be done with a folding 6× linen tester or a battery operated 10× hand lens but more detailed laboratory work would require at least 7× to 10× magnification. The finer particles in the same hammermilled feed would require 30× to 75× magnification. The color, luster, particle shape, particle size, effects of processing, etc. would be visible at the magnification of the stereomicroscope.

Seed coat particles, ground yew leaves, insects and insect fragments, mineral particles and many other particles associated with toxicants could also be examined and confirmed with the stereomicroscope. It is important to keep in mind the relative sizes of structures to be examined and the type of information one can obtain from each. For example, a macroscopic feed examination would involve those characteristics that can be defined by minimal magnification. The naked eye aided by a hand lens or magnifying glass provides sufficient magnification to determine the feed ingredient or formula feed color, shapes of particles and other very obvious physical characteristics. A finished feed might be described as a densified, polished feed pellet, an extruded star-shaped petfood form, a floating fish particle, a granular crumble of a relatively soft pelleted feed for chickens or perhaps a mixture of silage, whole corn and a concentrated protein pellet for feeder cattle. All of these could be done with a hand lens and a needle probe. Similarly, a suspected feed ingredient or material that accidentally got into a feed might be described by its source. A description could be "weeds along a confinement area with potential toxic seeds," or a note that an on-farm feed mill was using roasted soybeans recovered from a sunken barge. Perhaps late harvested corn with extreme weather damage or restaurant cooking oil with floor sweepings was being used. Every piece of the puzzle needs to be collected and considered because when toxicants get into feeds the situation becomes a forensic analysis.

The higher magnifications of a stereomicroscope would be needed to complete the above examinations. The shift from macroscopic to stereoscopic would allow a more detailed picture of the weathered corn, any potential mold contamination of the recovered waterlogged soybeans and the seed characteristics from the ingesta of a free roaming animal. The compound microscope plus some careful sample preparation would be needed to jump up to a cellular or histological analysis. The higher magnification would permit a visible examination of mold mycelia invading the seed structure, the cell types and sub-cellular calcium oxalate crystal inclusions of certain leaves and other definitive structures used for identification. The compound microscope permits the unique cellular characteristics of all the ingredients to be used for the analysis.

## CLASSIFICATION OF FEED INGREDIENTS BY SOURCES

Feed microscopy can also be sorted, studied and examined by the ingredient sources. This allows parallel

comparisons of similar structures which are related uniquely to each species. Each level of detail opens new opportunities for confirming an analysis.

*Processed feed grains, legumes and oilseeds* are whole seed sources that are minimally processed for feeds. Corn (maize) is one of the most common feed grains in the world. It is fed whole, ground or flaked. Grinding and steam flaking enhance the nutrient availability particularly of carbohydrates because corn is primarily a starchy energy source. The extent of starch gelatinization can be observed with polarized light. Grain sorghum and the several millets are smaller seeds that are fed in the same manner. Wheat, barley, rye, oats and rice are more typically food grains but lesser grades are used directly in feeds. The feed microscopist can identify ground particles from each of these sources by their inherent structural characteristics. For example, an examination of an unknown flat particle associated with intact starch could be compared to the modified leaf tissue that in grains is considered as chaff or hull. Wheat chaff is left in the field and is not in direct contact with starch in the seed. Therefore, logically the microscopist would search inwardly to the next flat layer under the hull which is pericarp. The outer pericarp, when ground, often remains attached to other layers of the original ovary wall plus the testa and aleurone layers and is called bran in commercial channels. The unknown would be compared to reference standards of the pericarp or bran for each cereal. If the initial observations that the particle was associated with starch were true, the particle would likely match the cell types and be confirmed as a bran particle of one of the cereals. Knowledge of the structure of all the seeds commonly used in feeds allows a logical comparative examination of any cereal or other feed ingredient.

An example of a confirmed minimal processed legume is cracked or whole fed soybeans. The soybean is the only major feed legume that is fed whole but it is usually roasted to minimize trypsin inhibitors. When whole soybeans are roasted, the seed coat splits transversely to the embryonic axis and remains on the seed. It is a unique characteristic associated with roasting. The extent of roasting could be measured by the residual urease activity. Other legumes such as lentils, lupine, dry beans and peas are found in feeds but not as mainline ingredients and they have no significant trypsin inhibitors that would require heat treatment. Each of these clues leads to a confirmed analysis.

Cottonseed is one of the few oilseeds fed whole as a fiber source in cattle. The seeds are very fuzzy and can be easily identified by the attached cotton fibers that rotate polarized light, have a dumbbell-shaped cross-section and exhibit a slow twist. In addition, the hull is a three-layered achene that is multicolored and unique to cottonseeds. Many of the characteristics exhibited by

oilseeds are similarly so specific that tiny particles of the oilseeds can be identified even after they have been hammermilled and processed into finished feeds.

*Processed feed grain, legume and oilseed byproducts* are food byproducts on which the entire feed industry is based. A wide range of ingredients from the production of foods are available. These include corn bran, corn gluten feed, corn gluten, hominy feed, wheat middlings, wheat bran, barley hulls, rice hulls, oat hulls, just to list a few. Legume and oilseed byproducts include soybean hulls, peanut meal and hulls, canola meal, linseed meal, sunflower meal and hulls, safflower meal and hulls, cottonseed meal and hulls, and many less common meals. Each ingredient has specific characteristics at each level of magnification to identify it with confirmation at the cellular level. These structures can be found in the listed references of this chapter or created by careful dissection of authenticated reference standards.

*Processed byproducts from other plants* include citrus pulp, bagasse from sugar cane and beet pulp from sugar beets, fruit pomaces, tomato pomace, potato meal, food gum meals and other food byproducts. Almost everything that is not designated human food finds its way into feeds.

*Processed plant products from industrial sources* include distiller's grains and solubles from the power alcohol and distillery industries, fats and oils from off-grade edible and detergent oil sources, *Aspergillus* meal from food and industrial enzyme production, and many others. The oils are particularly problematic because they have no visible cellular structures and can get mixed accidentally with miscible industrial liquids and pesticides.

*Processed animal products and byproducts* include whole animal meals such as chopped beef, fish meal or poultry, lamb or venison meat. Examples of meat meals include blended meat meal (beef and pork), fish meal from larger trimmed out or canned fish, and all manner of rendered meat products. Feed microscopy is currently the major firewall against BSE (Bovine Spongiform Encephalopathy) or mad cow disease tissues getting into cattle feed.

*Processed natural mineral products and metallic wastes* are the minerals in feed. On rare occasions, ground circuit boards and other electronic and industrial waste that contains metallic components get into feeds. Cadmium, arsenic, lead and other toxicants also find their way into feeds. These can be detected by the microscopist and suitable chemical testing initiated to determine the extent of the problems.

*Processed aquatic products and byproducts* include fish meals, shellfish meal, oyster shells, shrimp meal, shrimp shell meal and shrimp head meal, squid meal and other specialized products. Of all the byproduct meals, the aquatic ones are the most variable and potentially the most adulterated because they lack specific standard descriptions for trading. The feed microscopist is often quite challenged by these meals in feeds.

## CLASSIFICATION OF FINISHED FORMULA FEEDS BY PROCESSING

We can also classify finished feeds by the type of agglomeration and cooking processes used as an aid to understanding the effects of processing on the feed ingredients. Although the key identifiers will not change dramatically, it is important to understand that the extent of conditioning and moisture incorporation during processing will (1) affect the starch gelatinization and other binding reactions and (2) affect the methods used in the microscopic examination to reverse the hydrothermal bonds and permit easier examinations. Some of the common processes and applications are as follows.

*Pelleted and crumbled feeds* are used to maintain uniform nutritional distribution in feeds. The pelleting operation increases the bulk density and fixes the nutrients so that no segregation occurs during shipment and handling. The pellets are crumbled to make it easier for poultry and small animals to feed. Crumbling increases the surface areas for saliva or other digestive juices to be absorbed and to speed digestion. Crumbling also increases the available surface area for moisture absorption and in humid areas can lead to mold growth. Pelleted feeds have relatively friable structures due to minimum starch gelatinization. They can be readily hand ground in a mortar and pestle. The particles will usually separate adequately to conduct an analysis when aided by solvent flotation and sieving to separate fine particles and fat, and ease the identification of fine particles.

*Expanded and extruded feeds* enhance nutrient availability and feeding efficacy primarily by increased gelatinization during conditioning and the ability of the extruder to process higher moisture feed mixtures. The extruder combined with the expander, particularly when the feed is re-ground or re-extruded for aquaculture feeds and some pet foods, reduces the sizes of the individual feed particles to the point that the feed microscopist can barely identify the sources of the ingredients. The extent of gelatinization is much greater for extruded matrices and they do not separate easily even after mortar and pestle grinding, flotation and sieving. They are a much more difficult matrix to separate and examine.

*Steam conditioned and rolled feeds* have enhanced cell disruption and nutrient availability. The process is used primarily for whole grain corn and sorghum that are to be fermented or reconstituted and fed with silage and other rations. Unless flaked grain has been mixed with other ingredients, no additional separations are needed prior to the microscopic examination.

*Canned pet foods* are specialty soft and wet products with unique attractants and flavors. They require thermal processing to maintain shelf life stability and palatability. They are particularly difficult to analyze but are



commercially sterile and not prone to microbial growth or related toxicant problems.

### CLASSIFICATION OF FORAGES BY POINTS OF CONTACT OR POTENTIAL TOXICANTS

Lastly, we can classify feed resources by their natural habitats and their potential for toxicological problems. These plants and toxicant situations are not commonly seen by the feed microscopist but a good broad knowledge of plant structures makes the feed microscopist particularly well suited to examine them. These include native forages and pasture plants that may be consumed due to animals roaming free on pasture and via casual contacts. Some cut and stored forages and silages that are fed to confined animals may be weathered and spoiled. Exotic and introduced plants may be consumed due to invasive growth in pastures or by accidental cuttings and unknowledgeable feedings. Finally, decorative house plants and some garden materials may be consumed by pets. All these types of forage samples are examined as they are taken. That is, no specific methods are used to reduce the particle size because the evidence will be compromised by additional processing. Instead the particles are separated by hand into appropriate classes to be examined. For example, a whole kernel corn, silage and concentrate pellet feed would be separated on a large tray by hand or with minimal sieving depending on the particle size classes involved. Hand separations are generally easiest, although time consuming. The corn and pelleted concentrate would be dried and each examined separately. The pellet would be handled like any other pelleted feed and analyzed for its component parts. The silage would also be dried and, if a toxicant was suspected, the examination would focus on the purity of the silage including weedy and herbaceous forages, evidence of wild fermentation, mold growth and the extent of uniformity and the presence of additives. Identifications would be based on collected forages from the farm or ranch or keyed to range and pasture plant references. Unknown forages could also be identified through herbarium collections.

### IDENTIFICATION OF INGESTA PARTICLES

The identification of fecal and ingesta samples is very similar to the routine examinations of feeds and forages. Sometimes particles can be isolated directly from the samples. Otherwise the samples require pre-analysis

preparation for biological safety for the microscopist. A standard fume hood or air handling unit to isolate aromas and filter out dried dusty particles would also be needed for biological safety.

Digestion, even through a rumen, is similar to the feed manufacturing process and does not destroy the cellular characteristics used for identifications. Thus, the identification of yew or hemlock leaves, cockleburrs, crotalaria and other toxic weed seeds and most other plant toxin sources is the same as for forage examinations. Reference standards of the seeds and plant tissues are the key to the identifications, although having some ingesta samples for comparisons is always helpful. Medical laboratory manuals also provide routine methods for the safe handling of fecal sample examinations that can be applied to feed microscopy.

### THE ROLE OF THE ATTENDING VETERINARIAN

Because few feed microscopists are available for on-site examinations, the attending veterinarian should have an understanding of feed microscopy and what it can provide for an investigation. The veterinarian should be aware of the sampling requirements for microscopy, the need to secure both the feed ingredients and the finished feeds from bagged storage, from feed bunkers, from augers and feed mixing, handling and feeding systems, and from any other on-farm or confinement locations. Samples from baled hays or forages, silage bunkers, fence rows, pastures and other waste areas available to animals should be retained. In the case of pets and small animals, representative samples should include house plants, decorative plantings and garden areas around the house. Too often the feed microscopist is given only a sample of a suspect feed or ingredient which may or may not be related to the toxicant problem. A more encompassing view should be encouraged. Because sampling is one of the most critical aspects of any toxicant situation, samples representing the earliest possible point in time should be secured as quickly as possible and retained for microscopic examinations.

If an ingesta or fecal sample is available, it should be processed immediately or frozen (iced for short-time transport) at  $-10^{\circ}\text{C}$  or below to limit microbial activity. The following is a simplified analytical scheme to handle these samples.

*Ingesta or fecal samples* should be separated into the portion for chemical testing and the microscopy aliquot. A 5 or 10g wet weight aliquot can be rinsed and washed thoroughly through a 260 or 400 mesh stainless steel sieve to retain essentially all particulates but remove all the

solubles (assuming no chemical or biochemical testing will be needed on that sample aliquot for extra-cellular constituents). The particulate residues should be transferred from the sieve to a hard surfaced but porous filter paper (standard pleated coffee filters work well) and the sample air dried. Do not heat the sample if any enzyme-based testing will be required. The dried particles, particularly the fibrous cellulosic particles, may be gently rolled in a mortar with the pestle to cause the fibrous particles to separate. Do not attempt to grind them. If the sample is too fatty to separate and the particles cannot be identified due to a coating of fat, flotation on chloroform (observe the current laboratory safety procedures for the solvent) may be necessary as both a defatting step and a separation of mineral constituents and particulate fines. Defatting will take about three rinses using 2–3 times the volume of the fatty aliquot. When the chloroform has evaporated, the examination can proceed with standard feed microscopy methods comparing the unknown particles with reference standards and confirming the observations at the cellular level. Remember, most of the digestible nutrients will have been removed from the cellulosic cell matrix debris. This may change the appearance slightly but the cellulosic structures will not have changed, depending on passage time, and can be compared to reference standards of ingesta and feces if they are available. Animal tissues would have been digested and would not generally be available for examination.

## FEED MICROSCOPY TRAINING AND AVAILABLE LITERATURE

Although this chapter has briefly introduced feed microscopy and what it can do for toxicological examinations, some mention of where training is available, how one organizes a reference sample collection and sets up a laboratory may be of interest. Because feed microscopy is a “hands-on” practical technology, it is not easily learned from a book. It is instead taught as an intensive short course. An Internet search is currently the best way to locate feed microscopy short courses.

Feed microscopy draws on all the scientific disciplines and information exists but is widely scattered and often out of print and not readily available. The interpretive drawings of [Winton and Winton \(1932\)](#) still stand as one of the best reference sources ever published for food and feed structures. [Vaughan \(1970\)](#) updated the oilseeds with excellent interpretive drawings and some photographs. *The Manual of Microscopic Analysis of Feedstuffs* ([Bates et al., 1992](#)) draws the most important technical information together into a single reference book specifically for feed microscopy but it is not a textbook or a training manual. [Klein and Marquard \(2005\)](#) put

together an extensive *Atlas of Feed Microscopy* which provides many line drawings that had not been previously available in English. *The Aquaculture Feed Microscopy Manual* ([Bates et al., 1995](#)) explored the ingredients commonly used in fish, shrimp and other confined aquatic organisms. *Introduction to Food-Borne Fungi* ([Samson et al., 1995](#)) is one of the best references for detailed photos and interpretive drawings of the common molds attacking foods and feeds. What is most notable about the feed microscopy literature is the lack of a single treatise or detailed text for the feed microscopist that covers everything including the cellular keys for identification of forages and pasture plants and the identification of ingesta and fecal particles. Reference standard collections are critical to feed microscopy and ingesta identifications. Typically microscopists share samples or hard-to-find materials at professional meetings or via mailings. Sample collections of authentic materials are usually provided at feed microscopy short courses.

## THE FEED MICROSCOPY LABORATORY

Feed microscopists generally work alone, not in groups, and their laboratories are reflections of their work habits in whatever space is available. An ideal feed microscopy laboratory work station is centered in a “U” configuration with sinks, hoods, preparation areas, etc. located nearby but not in the immediate microscope area where fumes or solvents might damage the microscopes. [Figure 103.1](#) shows a laboratory with shallow drawer cabinets full of reference standards supporting the work surfaces holding the microscopes. A boom stand stereomicroscope is at the right side, a large research stereomicroscope is in the center equipped with video and



**FIGURE 103.1** An efficient “U”-shaped feed microscopy laboratory configuration.

still camera ports and a large frame compound microscope is seen on the left side. A microscope and a second compound microscope are just outside the picture borders along the legs of the "U." A balance, hand tools, reagents, reference materials and many other small items used in the analyses can be seen in the photo along with small cabinets for all types of reference standards.

## THE EQUIPMENT

The following equipment list details briefly the essential items plus many that are useful but may not be available in most laboratories. In all cases, purchase the best equipment that can be justified. Remember, many extremely fine used microscopes are available for the starting microscopist:

- *Stereoscope*: Research grade, zoom microscope equipped with a brightfield/darkfield base and trinocular head. The newer models provide more options for digital photomicrography, videography, illumination and depth of field than the older, more simplified microscopes. Because the stereomicroscope is the heart and soul of the equipment used, it should be selected carefully to do all the types of examinations that are anticipated.
- *Compound microscope*: Large frame research model. Many excellent used Leitz, Zeiss and other brands from the mid-1980s are still available and serviceable. Most microscopy laboratories cannot justify purchasing the latest research models.
- *Dual compound microscopes*: Bridged for side-by-side comparisons of unknowns and authentic reference standards.
- *Documentation*: Digital cameras can be readily interfaced with microscopes to provide a digital record of observations, reference standards, side-by-side comparisons, etc.
- *Hand tools*: This should include tweezers, needles and probes for manipulating samples, small cutting instruments (scalpel, micro-scissors), glassware and sieves for flotation separations, measuring tools (manual or digital calipers, optical and stage micrometers and measuring cells for particulates). Many microscopists fabricate their own tools or adapt and modify tools from other disciplines. Dentistry picks and probes, tools for clay sculptors, model builder's scissors, micro-soldering torches and many other small tools have been adopted for use under the microscope.
- *Reference standards set*: The collection of reference standards (authentic) is a critical aspect of feed microscopy. The reference collection is one of the central three requirements for microscopy and maintaining a collection is a career-long endeavor. Every effort

should be made to collect multiple samples of each feed ingredient to represent the variations from different parts of a country or different appearances due to heating, drying and other processing variations of manufactured byproducts. The underlying cellular structures will remain essentially the same (except for agglomerated and co-mingled byproducts) but the appearances may differ.

The reference collection should be preserved in glass or plastic containers that can be well sealed and maintained free of insects. Freeze potentially contaminated samples for 10–15 days before placing them in the collection. No pesticides or other chemicals should be used for preserving a reference collection because natural aromas are an inherent part of the ingredients.

## CONCLUSIONS AND FUTURE EXPECTATIONS

Feed microscopy offers a wide range of applications for the analysis of feed ingredients, finished feeds, ingesta and fecal samples. Although the classical applications of feed microscopy in the feed industry and state compliance laboratories have become relatively static due to a series of events and decisions ranging from load cell technology to least cost formulations to collective terms for labeling, the needs for microscopists in BSE screening and bio-security issues, toxicology and forensic applications have dramatically increased. Thus, this is a time of transition. This chapter illustrates just a fraction of the applications and opportunities for feed microscopists. The identification and evaluation of particulates from foods, feed ingredients, formula feeds, forages, weed seeds and all manner of other sources will continue to expand for many years if the current demand can be interpolated correctly into the future.

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## Prevention and treatment of poisoning

Camille DeClementi

### PREVENTION OF POISONING

Prevention of poisoning in household pets consists of controlling the animals' environment to decrease exposure to potentially dangerous substances. This requires animal caretakers to be diligent and knowledgeable of potential risks. While much of the advice offered to prevent poisoning will seem like common sense to many veterinarians, these guidelines are often unfamiliar to animal caretakers. Veterinarians are therefore encouraged to share this information with their clients.

Clients should be reminded to keep all veterinary and human medications, both prescription and over-the-counter (OTC), out of the reach of animals. Since some pets are able to climb onto high surfaces and open cabinets, medications are not adequately "out of reach" in those places. Owners should be instructed not to give their pets any medication, including their own, unless directed by their veterinarian. Clients may not realize that giving an OTC medication that they consider safe, like acetaminophen, could cause life-threatening illness in their pet. For example, treatment of alopecia in cats with topical minoxidil solution has led to pleural effusion, pulmonary edema and death (DeClementi *et al.*, 2004). Clients should also store all other potentially hazardous products, including cleaning products, auto-care products, pesticides and insecticides, out of the reach of their animals. Garbage cans should be sealed with tamper-proof lids.

Animal caretakers should be urged to read all label information before using a product on an animal or in the animal's environment and to follow the label instructions exactly. Veterinarians should mention that it is

often not safe to use a product on an animal species for which it is not intended. For example, using a concentrated permethrin flea product labeled for dogs could prove deadly if used on a cat (Richardson, 2000a).

Since many plants are poisonous, clients will want to be aware of the plants in an animal's environment, including those in outside areas. Additionally, they should be alert to any fluids leaking from vehicles and clean up leaks immediately. If a rodenticide or other bait is necessary in the home or yard, the product should be placed in an area that is completely inaccessible to non-target animals. The bait should be removed as soon as it is no longer needed. An animal's enclosure should be routinely checked, and unfamiliar or questionable items removed. Companion animals should be supervised, when possible, if they are outdoors, and a securely gated, confined area should be provided when animals are left unattended.

The guidelines for keeping household pets safe from poisoning are very similar to those for children, especially toddlers. Some pets may even be more at risk than children because, unlike children, they are commonly left unattended. They are also likely to chew open some containers, including those considered child-safe. The ASPCA website has additional information regarding poison prevention ([www.asPCA.org](http://www.asPCA.org)).

Decreasing the risk of exposure to toxicants is also important in large animals. Caretakers should be urged to purchase hay and feed from reputable suppliers, to examine the feed for recognizable contamination, and to verify cleanliness and high quality. Feed should be stored in a clean, insect-free area at the appropriate moisture level to prevent damage and lessen the risk of contamination or mycotoxin growth (Osweiler, 2001).



Learning to identify poisonous plants and their potential effects is critical for large animal clients. Once poisonous plants have been recognized in an area, many strategies can be employed to limit exposure. The most effective strategy is to remove animals from poisonous plant-infested areas either by herding or fencing off those areas. If this is impossible, attempts should be made to reduce the poisonous plant populations via mechanical (burning or pulling), chemical (herbicides), biological (such as using the larvae of the cinnabar moth to control tansy ragwort), or other control methods. Since treatment with herbicides may increase palatability or toxicity in some plants, animals should not be allowed access to herbicide-treated areas until all treated plant material is dead and removed. Once the plant populations are controlled, good grazing management can be used to maintain the area in a condition that limits regrowth of the plants (Cheeke, 1998).

Large animal enclosures and barns should also be kept free of other potential toxicants, including insecticides, pesticides, petroleum products and medicated feeds. Following label instructions is just as important for large animals as household pets. Medicated feeds, medications and insecticides should be used only on the labeled species. If a pour-on amitraz product intended for cattle is instead used on horses, fatal ileus may result (Gwaltney-Brant, 2004).

## STABILIZATION AND MONITORING

Toxicant exposures often require immediate or urgent attention. Television and movies have led the public to believe that every toxicant has an antidote; consequently animal caretakers may expect their veterinarian to provide one. Unfortunately, this expectation is far from reality. There are very few antidotes and when they do exist, they may be cost prohibitive or difficult to obtain. Therefore, it is critical for the clinician to concentrate on *treating the patient and not the poison*. Poisoning cases should be managed following the same principles of triage and patient stabilization as other emergencies. Decontamination and antidote administration, if available, should follow initial stabilization.

A detailed history should be taken after the patient is stabilized, but the following questions should be asked on presentation. To what toxicant was the animal exposed? What amount? When did the exposure occur? Has the patient shown any effects? Have any treatments been performed (for example, dilution, emesis or bathing)? Have other animals also been exposed?

As in any emergency case, initial evaluation and stabilization of the patient should address the basic ABCs (airway, breathing, bleeding, cardiovascular, circulation

and level of consciousness). The clinician must assure that the patient has an adequate airway and is not having difficulty breathing. If needed, an endotracheal tube should be placed or a tracheostomy performed to establish a patent airway. If the patient is dyspneic, 100% oxygen should be delivered via oxygen cage, mask or nasal cannula (Mathews, 2006). Oxygen supplementation is contraindicated in a paraquat exposure (Oehme and Mannala, 2006). Stress and handling should be minimized in a dyspneic patient.

The patient should be checked for bruising and for signs of active bleeding from the nose, mouth, anus and vulva or penis. The mucous membrane color and capillary refill time should be evaluated. Pulse rate, rhythm and strength should be assessed. Electrocardiogram (ECG) and blood pressure monitoring should also be performed. If needed, an intravenous (IV) catheter should be placed and fluid therapy initiated. Attempts should be made to control life-threatening arrhythmias (Mathews, 2006).

The patient's level of consciousness should be determined. If the patient is actively seizing, diazepam, given as an IV bolus at a dosage of 0.5–1 mg/kg, is often the initial drug used for dogs and cats. If this is not effective, other medications including phenobarbital, pentobarbital and propofol may be useful. Inhalant anesthesia may also be used to control the seizure activity (Mathews, 2006). The reader is referred to a formulary or reference on emergency seizure control for recommendations on doses and drug choices for the species being treated. Since some diagnostic tests, including that for ethylene glycol, may give false results once injectable medications are administered, the clinician should obtain blood samples prior to administering these medications. Body temperature should be checked and thermoregulation initiated if needed.

Once the patient has been stabilized, a more thorough clinical evaluation can be completed including complete history, physical examination and appropriate diagnostic testing (Cantilena, 2001), and a treatment plan developed. The signalment and health history of the patient is important in developing a treatment plan. If the patient is a nursing or pregnant female, for example, precautions will need to be taken to prevent exposure to the fetus or young. Or if the patient is taking a highly protein-bound medication, it is more at risk from an overdose of another protein-bound drug. If there is no known exposure to a toxicant but poisoning is suspected based on presentation and clinical findings, the questions in Table 104.1 may be helpful to reveal a cause.

Next, a complete treatment plan should be developed. It should include what method, if any, will be used to prevent further toxin absorption and if an antidotal therapy will be used. The plan should address any clinical signs not addressed in the initial stabilization. It should

TABLE 104.1 History gathering questions

- 
- When was the patient last normal?
  - How long have the signs been present?
  - Were there any initial signs that are no longer present?
  - Was the onset of the signs gradual or sudden?
  - Are there other pets in the area? Are they affected?
  - What was the location of the animal in the last few hours prior to development of signs? Was the animal supervised?
  - Is the animal indoors or outdoors mostly?
  - If the animal is outdoors, is it confined or does it roam?
  - To what areas of the home or garage does the animal have access?
  - Have any new foods or treats been introduced to the animal's diet?
  - Any access to sugar-free products?
  - Has there been any recent access to garbage?
  - Has the animal chewed or destroyed anything recently?
  - Are there any medications in the house (human, veterinary, prescription, OTC)?
  - Could the animal have been exposed to illicit drugs?
  - Are there children or teenagers in the household?
  - Have there been any recent visitors who may have dropped medication?
  - Are there any rodenticides or other baits being used in the home or yard?
  - Could the animal have ingested any plants (indoors or outdoors)?
  - Have any medications, herbal products or insecticides been administered to this or any other animals in the household recently?
  - Are there any mushrooms growing in the yard?
  - Is there a compost pile in the yard?
  - Have any yard treatments been applied recently?
  - Are there any livestock in the animal's environment?
  - Have any livestock been recently euthanized or buried on the property?
- 

list what additional findings may yet develop and a plan of action for each. The plan should define how often the patient will be monitored and what indices will be evaluated. And it should determine what diagnostic tests are appropriate for the situation. The treatment plan may need to be updated as the case progresses.

Diagnostic testing will be governed by clinical findings, history and, in a known exposure, expected effects. The clinician should perform baseline testing and repeat as required throughout the course of the treatment. Appropriate testing may include a packed cell volume (PCV) and total solids (TS) to identify dehydration or blood loss in cases of anticoagulant exposure or agents that may cause gastrointestinal (GI) ulceration. A complete blood count (CBC) may be needed to check for inflammation or secondary infections in cases where GI ulceration and perforation are possible, such as in exposures to cationic detergents, corrosive materials or non-steroidal anti-inflammatory medications (NSAIDs). Monitoring the CBC is also indicated in exposures that may cause bone marrow suppression such as estrogen overdoses.

A full chemistry panel is often appropriate. Blood glucose should be monitored closely with exposures to sulfonyleurea hypoglycemic agents and in exposures to the sugar-free sweetener xylitol in dogs. Monitoring the renal values, blood urea nitrogen (BUN) and serum creatinine is important in many exposures to identify dehydration and renal alterations if the patient was exposed to potential renal toxicants such as lilies in cats, grapes and raisins in dogs, NSAIDs and ethylene glycol. In these cases, urinalysis is also indicated. Electrolytes should be monitored in cases where IV fluid therapy is required and in cases where the toxicant may lead to electrolyte abnormalities. For example, hypokalemia is a common effect in dogs that puncture albuterol inhalers (Vite and Gfeller, 1994).

Monitoring of acid-base status is important in some cases, such as exposures to aspirin and ethylene glycol. The cardiovascular system will need to be monitored, via ECG and evaluating blood pressure in exposures to certain agents such as pseudoephedrine and the methylxanthines in chocolate. Other general testing including pulse oximetry, radiographs and ultrasound may be required depending on the toxicant. Specific tests, such as an ethylene glycol test or serum iron panel, may also be suitable depending on the situation.

In addition to these physiological parameters, the patient's clinical signs, vitals, behavior and mentation should be monitored frequently for the duration of the treatment and abnormalities addressed. The frequency and degree of monitoring will depend on the situation. The patient's attitude should be noted regularly. Respiratory and cardiovascular status should also be checked at regular intervals, especially in cases where these systems may be affected by the toxicant. Capillary refill time and mucous membrane color can be used to assess peripheral perfusion. Body temperature should be monitored if the toxicant, clinical findings or treatments may cause abnormalities. Appetite, hydration status, bowel movements and urinary output should also be noted.

## DECONTAMINATION

Once the patient has been stabilized, decontamination should be considered to prevent additional exposure to the toxicant. Although the basics of decontamination are similar among species, the specific method of decontamination that is chosen in each case must be guided by the exposure circumstances and the species exposed. For all decontamination methods, consider sedation or anesthesia if the procedure will be very stressful for the patient, but only if the health of the patient will allow. The handler should wear appropriate protective clothing

including gloves, mask and eye gear to prevent personal exposure. Most exposures to toxicants can be broken down into ocular, dermal and oral exposures. The following are methods of decontamination for each of these exposure types.

### Ocular exposure

Ocular exposures may cause effects ranging from mild irritation to corrosive injury and blindness depending on the substance, the concentration, the exposure time and the sensitivity of the patient. With any ocular exposure, the eyes should be flushed repeatedly with tepid tap water or saline solution for a minimum of 20–30 minutes (Rosendale, 2002). An eyedropper can be used for smaller patients like birds or reptiles. With a larger patient, fill a plastic cup and slowly pour the contents over the ocular area. Patients can be given a mild sedative prior to flushing if needed and if the health of the patient will allow. If not sedated, the patient should be allowed to rest at regular intervals during the flushing to minimize stress. Fluorescein staining should be performed after flushing and again at 12–24 hours post-exposure to check for corneal ulceration. Additional treatment with ophthalmic and systemic medications may be necessary.

### Dermal exposure

Dermal exposure to a large variety of substances, including petroleum products, pesticides and insecticides, corrosive or irritating materials and substances that are sticky (tar, asphalt, sap and glue), may occur. Removal of such substances may be less stressful for the patient and safer for the handler if sedation is used. Sedatives should only be used if the health of the patient will allow. If not sedated, the patient should be allowed to rest at regular intervals to minimize stress.

In birds, dermal substances can be removed by using a water bottle to spritz the bird lightly with room temperature water. This procedure should be done in a warm environment to prevent chilling. The bird should be misted until the feathers no longer smell or feel of the product. If misting alone does not remove the product, and soap is needed, a liquid dishwashing detergent (e.g., Dawn™) may be diluted in the bottle and applied. After removal of the substance, the bird should be rinsed via misting with plain water until all soap is removed. In cases of heavy exposure, birds may be bathed with liquid dishwashing detergent and rinsed well. After misting or bathing, the bird should be wiped with a dry towel and kept in a warm environment away from drafts until completely dry. The procedure for reptiles is similar.

For dermal exposures in mammals, bathing in a mild liquid dishwashing detergent and warm water is recommended. Baths may be repeated in order to remove the toxicant completely (Rosendale, 2002). The animal should then be rinsed well with warm water and towel dried, then kept in a warm environment until completely dry. For smaller patients, like cats, that resent being sprayed with water, the bucket technique may be helpful. Fill a bucket with warm soapy water and, while supporting the hind legs, immerse the patient up to the neck. Remove the patient and continue washing. Use a fresh bucket with plain warm water to rinse well.

If the patient has a sticky substance on its fur, feathers or skin, do not use solvents to remove it since these may be irritating or corrosive to the patient. Instead, to remove sticky substances from mammals, remove as much of the substance as possible by trimming the fur. Then work a small amount of vegetable oil, mineral oil, mayonnaise or peanut butter through the rest of the substance until it breaks down into “gummy balls.” Afterwards, wash with liquid dishwashing detergent as described above (Rosendale, 2002). For birds, do not trim the feathers, just use vegetable oil, mineral oil, mayonnaise or peanut butter and then mist as described above.

### Oral exposure

When a patient is exposed to a potentially dangerous substance by ingestion, the clinician has many options for decontamination including dilution, induction of emesis, lavage, removal via endoscopy or gastrotomy, use of adsorbents, cathartics and administration of enemas. Often, the best treatment plan will include more than one of these methods.

#### Dilution

Dilution with a small amount of milk or water is recommended in cases where irritants or corrosive materials have been ingested. A suggested dose is 2–6 ml/kg (Mathews, 2006) which would be approximately only 1–2 teaspoons in an average-sized cat. It is important to use only a small amount of liquid for dilution. Using excessive amounts could lead to vomiting and re-exposure of the esophagus to the damaging material. For birds and reptiles, juicy fruits and vegetables can be fed to accomplish dilution. Dilution is not recommended in patients who are at an increased risk for aspiration, including those who are obtunded (Rosendale, 2002) or actively seizing. Additionally, milk, yogurt and cottage cheese have been useful as demulcents in cases of oral irritation following ingestion of plants containing insoluble calcium oxalate crystals (Philodendron species, for example) (Means, 2004b).

## Emetics

Emetics are usually most effective if used within 2–3 hours post-ingestion (Rosendale, 2002) but in some instances emesis may be effective even after that time frame. For instance, if a timed-released medication was ingested or if the substance ingested could coalesce to form a bezoar in the stomach, emesis may be effective later than 3 hours after the ingestion. Chocolate (Albretsen, 2004) and chewable medications may form bezoars. If the patient has not eaten in the previous 2 hours, feeding a small moist meal before inducing vomiting can increase chances of an adequate emesis. Emetics generally empty 40–60% of the stomach contents (Beasley and Dorman, 1990).

Dogs, cats, ferrets and potbelly pigs are examples of domestic animals that are able to vomit safely. Emetics should not be used in rodents, rabbits, birds, horses and ruminants. Rodents are unable to vomit (Plumb, 2005) and rabbits have a thin-walled stomach putting them at risk for gastric rupture if they vomit (Donnelly, 2004). It is not safe to induce emesis in birds, horses or cattle.

Induction of emesis is contraindicated with ingestion of alkalis, acids or other corrosive agents. When one of these products is swallowed, the protective epithelial lining of the esophagus may be damaged. This damage can leave the muscular layer of the esophagus exposed and at risk for ulceration, perforation and scarring if vomiting does occur (Beasley and Dorman, 1990). Emesis is not recommended after ingestion of petroleum distillates due to the risk of aspiration. Pre-existing conditions of the patient that can cause vomiting to be hazardous, such as severe cardiac disease or seizure disorder, must also be taken into account by the clinician when deciding whether to induce emesis. In all instances, the attending veterinarian must weigh the benefits of emesis against the risks. Emesis should not be attempted if the animal has already vomited or is exhibiting clinical signs such as coma, seizures or recumbency, which make emesis hazardous. Additionally, if the patient has ingested a CNS stimulant and is already agitated, the additional stimulation of vomiting could elicit seizures (Rosendale, 2002).

Hydrogen peroxide, apomorphine hydrochloride and xylazine hydrochloride are commonly used emetics in the veterinary clinical setting. Preliminary data obtained from the ASPCA Animal Poison Control's toxicology database indicate that hydrogen peroxide and apomorphine are effective emetics in dogs. Emesis was successful in 92% of dogs when administered either 3% hydrogen peroxide or apomorphine. No significant adverse effects were reported in dogs after emetic use. Apomorphine was poorly effective as an emetic in cats. Xylazine was an effective emetic in 57% of cats. When emesis was successfully induced, 68% of patients vomited some portion of the ingested toxicant (Khan *et al.*, 2009).

*Hydrogen peroxide*, at a 3% concentration, is a useful emetic when given orally. It is an ideal emetic for household use because the 3% concentration is used in many households for cuts and scrapes. Additionally, it is inexpensive and easy to administer. Hydrogen peroxide is thought to induce emesis via gastric irritation (Peterson, 2006). The dosage is 1–2 ml/kg (Beasley and Dorman, 1990) generally not to exceed 50 ml for dogs and potbelly pigs and 10 ml for cats and ferrets (Peterson, 2006). The dose can be administered with a syringe or turkey baster or can be mixed with a small amount of milk or ice cream to entice voluntary ingestion. Vomiting usually occurs within 10–15 minutes and the dose can be repeated once more if emesis is not initially successful. Walking or other gentle movement may be beneficial in initiating emesis.

*Apomorphine hydrochloride* is often used in the clinical setting to induce emesis in dogs. Use of apomorphine is considered controversial in cats since a safe dose has not been established (Plumb, 2005) and it is not very effective (Khan *et al.*, 2009). Apomorphine hydrochloride is a synthetic opiate that stimulates the dopamine receptors in the chemoreceptor trigger zone to cause emesis. The dosage is 0.04 mg/kg intravenously (IV) or intramuscularly (IM). Emesis is expected rapidly following IV administration, but may take 5 minutes with IM administration. Apomorphine can also be used conjunctivally by crushing and dissolving a portion of a tablet in a few drops of water. The solution is then administered into the conjunctival sac at a dose of 0.25 mg/kg (Plumb, 2005). This route may be preferred since the eye can be rinsed after emesis has occurred to prevent additional systemic absorption and decrease the likelihood of adverse events. At doses used to induce emesis, adverse effects may include central nervous system (CNS) depression and protracted vomiting. CNS stimulation and respiratory and cardiac depression may be seen with excessive doses (Plumb, 2005). The opiate antagonist naloxone can be used to reverse the CNS and respiratory effects, but will not block the emetic effect (Rosendale, 2002).

*Xylazine hydrochloride* is a potent  $\alpha_2$ -adrenergic agonist used in the veterinary clinic setting primarily as a sedative. It has also been used, with some success, as an emetic in cats (Beasley and Dorman, 1990; Khan *et al.*, 2009). Xylazine does not produce predictable emesis in dogs (Plumb, 2005). The recommended emetic dose in cats is 0.44 mg/kg IM. This is lower than the dose used for sedation in cats. Emesis is expected within 5 minutes of administration. Possible adverse effects include CNS and respiratory depression, hypotension and bradycardia. These effects, as well as the emetic effects (Beasley and Dorman, 1990), can be reversed by giving an  $\alpha_2$ -adrenergic antagonist, either yohimbine at a dosage of 0.5 mg/kg IV or atipamezole at a dosage of 50 mcg/kg IM (Plumb, 2005).



### Lavage

Lavage is sometimes used in cases where emesis is contraindicated, not possible or has been unsuccessful. If the patient is agitated, seizing or recumbent or has other health concerns, such as recent abdominal surgery, that increase the risks associated with induction of emesis, lavage is an option. Lavage should also be considered in species, like rabbits and rodents, which are unable to vomit safely. Lavage is unlikely to remove as much ingested toxicant as emesis (Beasley and Dorman, 1990) and is associated with significant potential risks. For these reasons, it should not be chosen haphazardly as a decontamination method over emesis. Lavage should also not be used to remove caustic substances or volatile hydrocarbons for the same reasons emesis is contraindicated in such cases (Rosendale, 2002).

Gastric lavage can be used in mammals to remove recently ingested toxicants. If the patient is a species with cheek pouches, the cheek pouches should be emptied gently with a finger or swab prior to the lavage. In all instances, a cuffed endotracheal tube should be in place to prevent aspiration, therefore general anesthesia must be performed unless the patient is comatose. Body temperature water (5–10 ml/kg) should be instilled via a large bore gastric tube with a fenestrated end, inserted to a length equal to the distance from the nose to the xiphoid cartilage (Beasley and Dorman, 1990). The head of the patient should be kept lower than the chest throughout the procedure. Gravity should be used to instill the water by holding the tube higher than the patient and then drained by moving the tube lower than the patient. The flushing process should be repeated multiple times (15–40) until the lavage fluid runs clear. With each flush, approximately the same amount of fluid instilled should be removed. The free end of the tube should be occluded before removal to prevent aspiration (Rosendale, 2002). The initial washings should be saved for toxicological testing if needed (Peterson, 2006).

In humans, hypernatremia following lavage with normal saline and hyponatremia following lavage with water have been reported. Additionally, one human study showed that as much as 25% of the fluid used for lavage passed into the small intestine. Thus, there is a concern that lavage may actually propel a toxicant into the small intestine where the absorptive surface area is greater. These risks should be limited by allowing the entire amount of fluid to drain out after each flush and by using only gravity to instill the water. Other risks associated with gastric lavage include esophageal or stomach damage or perforation, hypothermia and the accidental placement of the tube in the trachea and the instillation of fluid into the lungs (Rosendale, 2002).

A lavage technique can also be used in birds to remove recently ingested toxicants from the crop. To prevent

injury to the patient and handlers, anxious and fractious birds should be anesthetized prior to the procedure. If the patient is under general anesthesia, an endotracheal tube should be placed to protect the airway (Richardson *et al.*, 2001). The patient should be held with the head up and the mouth held open. The bird's head and neck should be extended to minimize esophageal damage. An appropriately sized feeding tube (soft plastic or rubber) is then passed into the crop. The crop should be palpated to assure correct placement. Then 10–20 ml/kg of warm saline is infused into the crop, the crop is massaged gently and the liquid is aspirated. The first washing can be kept for toxicological evaluation if needed. The cycle is repeated three or four times (Echols, 2005).

### Adsorbents

Adsorbents may be utilized in addition to or instead of emesis or lavage to prevent further systemic absorption of a toxicant. These agents act by adsorbing to a chemical or toxicant in the upper gastrointestinal tract and facilitating its excretion via the feces. The most commonly used adsorbent is activated charcoal. In the past, kaolin-pectin (Kaopectate®) had also been recommended as an adsorbent and demulcent in some instances. However, Kaopectate®, and most generic kaolin-pectin combinations, now contain bismuth subsalicylate as the active ingredient instead of kaolin and pectin. Some commercial-activated charcoal products also contain kaolin (Vet-A-Mix, Toxiban®).

Activated charcoal is composed of large porous particles that are able to adsorb and therefore trap a wide range of organic compounds within the gastrointestinal tract. The surface binding area of these products is quite large, in the range of 900–1500 m<sup>2</sup>/g (Rosendale, 2002). Charcoal tablets and capsules, found in pharmacies and used to control flatulence and bloating, are not likely to be as effective as the commercially prepared products (Buck and Bratich, 1986) as the concentration of the charcoal is often low and may have a smaller binding area.

The recommended dose of activated charcoal for all species of animals is 1–3 g/kg (or 1–3 mg/g) body weight (Buck and Bratich, 1986). There are many products on the market, including both liquid and powder forms. If using the powder formulation in dogs and cats, the total calculated dose of activated charcoal is mixed with 50–200 ml of warm tap water to make a slurry (Rosendale, 2002). In horses and ruminants, each gram of activated charcoal powder is mixed with 3–5 ml of water (Plumb, 2005). In symptomatic small animal patients and large animals, the activated charcoal liquid or prepared slurry is administered via stomach tube (Bailey and Garland, 1992). Small animals receiving activated charcoal via stomach tube should be sedated and have a cuffed endotracheal tube in place to prevent aspiration.

In small animal patients where no clinical effects are present, activated charcoal can be given orally with a large syringe or can be mixed with a small amount of canned food or chicken broth and offered to the patient (Rosendale, 2002). Some patients, especially dogs, will voluntarily ingest the mixture. Many birds will regurgitate a portion of the activated charcoal dose given and some dogs and cats will vomit after administration.

Repeated doses of activated charcoal may be indicated in some instances, such as cases where toxicants undergo enterohepatic recirculation. The first step in this process involves the toxicant being carried to the liver by either the portal vein after absorption from the gastrointestinal tract or via the systemic circulation. Once in the liver, the toxicant then enters the bile and is excreted into the gastrointestinal tract where it is again available for absorption. Many toxicants are known to undergo this type of recycling, including ibuprofen, marijuana and digoxin.

When repeated doses are indicated, half the original dose should be given at 4 to 8 hour intervals, often for 2–3 days (Peterson, 2006). It is important to mention that with medications that are excreted in the bile, activated charcoal can be of benefit regardless of the route the medication was administered. Thus, if a patient received an overdose of injectable ivermectin subcutaneously, activated charcoal will still be a very valuable decontamination option. The ivermectin molecules will be carried to the gastrointestinal tract by the bile.

The use of activated charcoal does carry some risks and it does not bind all compounds equally. Some chemicals that are not effectively adsorbed include: ethanol, methanol, fertilizer, fluoride, petroleum distillates, most heavy metals, iodides, nitrates, nitrites, sodium chloride and chlorate. Activated charcoal should not be given to animals that have ingested caustic materials since it is unlikely to bind them, it can be additionally irritating to the mucosal surfaces and make visualization of oral and esophageal burns difficult (Buck and Bratich, 1986). If ethylene glycol testing will be performed, activated charcoal should be administered after blood is collected, since propylene glycol found in many formulations can cause a false positive on some of these tests. Additionally, the timing of the activated charcoal administration should be taken into account when deciding on dosing of other oral medications since the charcoal can also bind them. In acetaminophen overdose in humans, for example, it is recommended that *n*-acetylcysteine not be administered orally until 3 hours after activated charcoal (Plumb, 2005).

Administration of activated charcoal carries a significant risk of aspiration. If the patient does aspirate the charcoal, the prognosis is poor. Hence proper placement of the stomach tube and a protected airway is a must in symptomatic patients. The patient may also experience constipation and black bowel movements, making

it difficult to determine if melena is present. If the activated charcoal sits within the gastrointestinal tract for a significant period of time, it may release the compound it has adsorbed, leading to systemic absorption and recurrence of the clinical signs. It is for this reason that activated charcoal is frequently administered with a cathartic. In fact, many commercially available preparations do contain a cathartic such as sorbitol.

Another possible adverse effect of activated charcoal administration is the development of hypernatremia. In humans, hypernatremia has been reported primarily in children when multiple doses of a charcoal-sorbitol mixture were administered. The hypernatremia is attributed to a water shift from the intracellular and extracellular spaces into the gastrointestinal tract as a result of the osmotic pull of the sorbitol cathartic (Allerton and Strom, 1991). The ASPCA Animal Poison Control Center (APCC) has also received reports of elevated serum sodium following activated charcoal administration in dogs. Hypernatremia seems to be more often reported in small dogs receiving multiple doses of activated charcoal, but it has also been reported in large dogs and in cases receiving only a single dose. Furthermore, unlike the human reports, hypernatremia has also been noted in cases where no cathartic was present in the charcoal product given (APCC unpublished data). Perhaps one of the other components of the product is also osmotically active. In these cases, the APCC has found that administration of a warm water enema is very effective at lowering the serum sodium and easing the resultant central nervous system effects.

### Cathartics

Cathartics enhance elimination of substances, including activated charcoal, by promoting their movement through the gastrointestinal tract. Since activated charcoal only binds to toxicants by weak chemical forces, without cathartics, the bound toxicant can eventually be released and reabsorbed (Rosendale, 2002). When used with activated charcoal, the cathartic should be given immediately following or mixed with the charcoal. Cathartics are contraindicated if the animal has diarrhea, is dehydrated, if ileus is present, or if intestinal obstruction or perforation are possible (Peterson, 2006).

There are bulk, osmotic and lubricant cathartics. The most commonly used bulk cathartic is psyllium hydrophilic mucilloid (e.g., Metamucil®). Psyllium is found in the ripe seed coatings of *Plantago* species. It absorbs water and swells in the intestine increasing bulk to induce peristalsis and decrease gastrointestinal transit time. The dose in dogs is 1 teaspoon to 2 tablespoons, and the dose in cats is 1–4 teaspoons (Plumb, 2005). Psyllium can also be used in birds by mixing one-half teaspoon with 60ml of baby food to form a gruel,

then administering by a dosing syringe or eyedropper (Richardson *et al.*, 2001).

Another bulking cathartic that can be used in dogs and cats is unspiced canned pumpkin. Dilute peanut butter, fruit or vegetables can also be used as bulking cathartic agents in birds and reptiles. Timothy hay can be utilized in rabbits. Bulking cathartics are also used to assist the passage of physical agents through the gastrointestinal tract (Beasley and Dorman, 1990). Examples include small pieces of plastic, coins, button batteries and sand in horses (Plumb, 2005).

Osmotic cathartics have limited absorption from the gastrointestinal tract so they are able to pull electrolyte-free water into the gastrointestinal tract, thereby increasing the fluid volume and stimulating motility to hasten expulsion in the feces. There are saline and saccharide osmotic cathartics. Sorbitol is the most commonly used saccharide osmotic cathartic; it is the cathartic of choice and is frequently combined with activated charcoal in commercially prepared charcoal products. The dose is 1–2 ml/kg of a 70% solution (Peterson, 2006). Sorbitol can be utilized in mammals, birds and reptiles.

The saline cathartics include sodium sulfate (Glauber's salts) and magnesium sulfate (Epsom salts). The recommended dose is 250 mg/kg. The use of magnesium sulfate has led to hypermagnesemia in some cases, which presents as depression of the central nervous and cardiovascular systems (Rosendale, 2002). Saline cathartics should not be used in patients with renal insufficiency or in birds or reptiles.

Of the lubricant cathartics, mineral oil is the most often used. Heavy mineral oil is preferred to light mineral oil since it is thought to carry less of a chance of systemic absorption and aspiration following oral administration. Mineral oil lubricates fecal material and the intestinal mucosa easing elimination and reduces absorption of water from the gastrointestinal tract, increasing fecal bulk and cutting transit time (Plumb, 2005). Mineral oil is not recommended as a cathartic following activated charcoal administration as the mineral oil may render the charcoal less adsorptive (Buck and Bratich, 1986; Galey, 1992). Mineral oil has been recommended to bind lipid-soluble toxicants including metaldehyde, nitrate and cantharidin (Stair and Plumlee, 2004; Plumb, 2005) and is used to treat impaction in horses (Buck and Bratich, 1986). The recommended dose in horses is 2–4 L per 500 kg body weight (Brown and Bertone, 2001). Possible adverse effects include diarrhea and aspiration of the oil leading to lipid pneumonitis. Ensuring proper placement of the stomach tube and slow administration lessen the risk of aspiration (Plumb, 2005).

As mentioned above, hypernatremia attributed to sorbitol administration has been reported in humans. Because all cathartics alter the water balance in the gastrointestinal tract, electrolyte abnormalities, especially hypernatremia,

are a potential risk to their use. A patient's hydration status should be monitored frequently and fluids administered, intravenously or via an enema, as needed.

### Enemas

Enemas are also appropriate when elimination of toxicants from the lower gastrointestinal tract is desired (Beasley and Dorman, 1990). Many extended-release or controlled-release medications are absorbed from the entire gastrointestinal tract, including significant absorption from the colon (Buckley *et al.*, 1995). Enemas can be used to move those medications quickly through the colon and lessen additional systemic effects. The general technique is to use plain warm water or warm soapy water. Commercial phosphate enema solutions should be avoided due to the risk of electrolyte and acid-base disturbances (Beasley and Dorman, 1990). Enemas are not recommended for birds since they already have a rapid gastrointestinal transit time. In reptiles, enemas may be useful since ingested materials often lag for prolonged periods in the colon.

### Endoscopy/Gastrotomy

In some cases, endoscopy or gastrotomy may be indicated to prevent further clinical effects. Endoscopy can be used to remove small objects, such as pennies, lead paint chips and small batteries. These objects can also be removed via gastrotomy if endoscopy is not available. Gastrotomy is also used to prevent obstruction from expanding foreign objects. Expandable isocyanate-containing glues (i.e., Gorilla Glue™) can swell once ingested to fill the entire volume of the stomach. A gastrotomy is needed in these cases to remove the mass (Horstman *et al.*, 2003).

## ANTIDOTAL THERAPIES

If an antidote to the toxicant a patient has been exposed to exists, the appropriate time to administer it will depend on the situation. In some instances, the antidote will be crucial in stabilizing the patient. For example, atropine should be used immediately in cases of organophosphate toxicosis in which the patient has life-threatening bradycardia and bronchial secretions (Gwaltney-Brant and Rumbeiha, 2002). On the other hand, in some cases, administration of the antidote is best done later. This is true for vitamin K<sub>1</sub> being used in anticoagulant rodenticide exposures. In a recent exposure, decontamination first would be the most appropriate course of action. In an anticoagulant patient that is symptomatic, supportive measures like a blood transfusion should be started first

since the vitamin K<sub>1</sub> will not be immediately life-saving (Merola, 2002).

Unfortunately, few antidotes exist. There is little economic incentive for pharmaceutical companies to seek approval for antidotal medications with only a small projected market (Post and Keller, 1999). Additionally, organizing clinical trials for antidotal medications is uniquely problematic. The U.S. Food and Drug Administration (FDA) has offered incentives to develop antidotal therapies through the Orphan Drug Act. As a result, at least one antidotal therapy was released for veterinary patients: fomepizole was approved for use in treating ethylene glycol intoxication (Cantilena, 2001). In situations where an antidote does exist, its use may be limited by its expense or availability.

Antidotes are generally divided into three groups, based on the mechanism by which they are protective. The groups are chemical antidotes, pharmacological antidotes and functional antidotes. Chemical antidotes act directly on the toxicant. They may decrease the toxicity of the agent or increase its excretion. Pralidoxime chloride, for example, does both; it binds to organophosphate insecticide molecules making them unable to bind to their target, and the pralidoxime–insecticide complex is then readily excreted (Mowry *et al.*, 1994).

Pharmacological antidotes antagonize the poison at the target site. Flumazenil, for example, has a high affinity for benzodiazepine receptors, thus it competes with benzodiazepines to reverse their depressive effects (Gwaltney-Brant and Rumbeih, 2002). Functional antidotes act to lessen the symptoms of the poisoning. They have no interaction with the toxicant. An example of a functional antidote is the use of methocarbamol to control fasciculations and tremors associated with tremorgenic mycotoxins (Schell, 2000). Below are some examples of antidotal therapies available for use in veterinary medicine.

### Antidotes for amitraz: atipamezole and yohimbine

Amitraz, a synthetic formamidine pesticide, is used topically to control ticks, mites and lice on cattle, pigs and dogs, as well as to treat demodectic mange in dogs (Grossman, 1993). It is also available in a collar form for tick control in dogs. The acaricide action of amitraz is not well understood, but it may have effects on the central nervous system of susceptible organisms. It also exhibits significant alpha-2 adrenergic agonist activity (Plumb, 2005). This activity is believed to be responsible for the clinical signs associated with amitraz toxicosis including ataxia, central nervous system depression, hypotension, hyperglycemia, mydriasis, hypothermia, gastrointestinal stasis and bradycardia. In dogs, toxicosis can occasionally result from exposure to a topical product but is more

commonly due to ingestion of an amitraz-containing collar (Grossman, 1993; Gwaltney-Brant, 2004).

*Atipamezole* (Antisedan®) is alpha-2 adrenergic antagonist labeled for use as a reversal agent for the sedative medetomidine. It has been used successfully in the treatment of amitraz toxicosis to reverse the central nervous system depression, bradycardia, gastrointestinal stasis and hyperglycemia (Grossman, 1993; Gwaltney-Brant, 2004). The recommended dose in dogs is 50 µg/kg IM. The drug has a good margin of safety; dogs injected with 10 times the therapeutic dose did not have significant effects. Potential adverse effects of atipamezole administration include vomiting, diarrhea, hypersalivation, a brief period of excitation or apprehensiveness and tremors (Plumb, 2005). In cases where an amitraz collar was ingested and has not been retrieved from the gastrointestinal tract, the atipamezole dose may need to be repeated each time signs recur until the collar is passed through the gastrointestinal tract.

*Yohimbine* is another alpha-2 adrenergic antagonist that has been used to counter the toxic effects of amitraz. The recommended dose in dogs is 0.1 mg/kg IV. Yohimbine has a short half-life (1.5–2 hours in dogs); consequently, the dose may need to be repeated. Potential adverse effects include temporary apprehension or central nervous system excitation, tremors, hypersalivation, elevated respiratory rate and hyperemic mucous membranes. Careful dosing is recommended, because tremors and seizures have been reported in dogs receiving five times the therapeutic dose (Plumb, 2005).

### Antidotes for anticholinesterase agents: atropine and pralidoxime

*Atropine* is used to counter the muscarinic effects of anticholinesterase agents. Acetylcholine (ACh) is a neurotransmitter that transmits impulses at cholinergic nerve synapses and neuromuscular junctions. The enzyme anticholinesterase (AChE) is responsible for the hydrolysis of ACh. Anticholinesterase agents, which include the carbamate and organophosphate (OP) insecticides, produce their effects by binding with AChE to disrupt the breakdown of ACh. The ACh accumulates within the synapse leading to overstimulation of the end organ (Meerdink, 2004). There are two basic cholinergic receptor types: muscarinic and nicotinic. The muscarinic are the postganglionic parasympathetic receptors in smooth muscle, the heart and endocrine glands. Overstimulation of these receptors leads to a complex of signs, often described as SLUDGE, which includes salivation, lacrimation, urinary incontinence, increased peristalsis and diarrhea, increased bronchial secretions and dyspnea, miosis, nausea, emesis and abdominal discomfort. Excessive stimulation of the nicotinic receptors, which are found in the



skeletal muscles and autonomic ganglia, leads to tremors, and possibly seizures, potentially followed by muscle fatigue, weakness and paralysis (Meerdink, 1989).

Atropine competes with the accumulated ACh in the synapse to block only the muscarinic effects. Atropine does not block the nicotinic effects. The dosage used to counter anticholinesterase agents is: dogs and cats 0.2–2 mg/kg; cattle 0.5 mg/kg; horses 0.22 mg/kg. In all of those species, one-quarter of the dose is given IV and the remainder intramuscularly (IM) or subcutaneously (SC) (Plumb, 2005). In some cases, administration of atropine may need to be repeated, but great care should be exercised to prevent over-atropinization. Auscultation should be performed to monitor the patient for bradycardia and continued bronchial secretions, since these are the most life-threatening of the muscarinic signs. Additional atropine should only be given if these signs are present. The patient will not die from miosis or hypersalivation. Horses are quite susceptible to ileus caused by atropine administration, and a total dose of no more than 65 mg is recommended for a horse of average weight (Meerdink, 2004).

Atropine is not an effective antidote for other types of insecticides including pyrethroids. When presented with a suspected case of anticholinesterase exposure, the clinician can use a test dose of atropine to assist in making a preliminary diagnosis. The patient should be given the preanesthetic dose of atropine (0.02 mg/kg) IV. If this dose is able to produce typical anticholinergic signs such as mydriasis and tachycardia, then the patient has likely *not* been poisoned by an anticholinesterase agent (Fikes, 1990). If the patient truly had been poisoned by an anticholinesterase agent, the dose of atropine required to produce those effects would be at least 10 times higher (0.2 mg/kg).

*Pralidoxime chloride* (2-PAM) is used with atropine in the treatment of OP poisoning to relieve nicotinic signs such as tremors and muscle weakness (Fikes, 1990). 2-PAM reactivates the acetylcholinesterase enzyme (AChE) that has been inactivated by the OP. Normally, acetylcholine (ACh) binds to the enzyme at the anionic binding site. OPs and carbamates bind nearby on the esteric site, thus physically blocking the anionic site from ACh and inactivating the enzyme. 2-PAM is able to squeeze in via nucleophilic attack and bind to the anionic binding site. It then attaches to the OP forming a pralidoxime–OP complex. This complex detaches from the enzyme reactivating it and is then excreted in the urine (Fikes, 1990; Mowry *et al.*, 1994; Plumb, 2005).

Administration of 2-PAM is most effective if given within 24 hours of exposure (Plumb, 2005). If the OP remains attached to the AChE much longer, aging of the bond may occur so that it can no longer be broken by 2-PAM (Meerdink, 1989; Mowry *et al.*, 1994). There are instances when later administration is warranted. For example, in large OP exposures, pralidoxime may still

be of some benefit if given within 36–48 hours (Plumb, 2005). And 2-PAM may still be indicated even later if clinical signs have been present for an extended period of time (Fikes, 1992). Please see Table 104.2 for dosing information.

Treatment with 2-PAM should continue until the animal is asymptomatic. If no improvement is seen after 24–36 hours following initiation of treatment, 2-PAM should be discontinued (Fikes, 1990). In acute feline chlorpyrifos toxicosis, cats with persistent tremors can be maintained on 1–2 times daily treatment for up to 4 weeks. The typical presentation in these cats differs from the classical signs expected from other OPs. The onset may be delayed 1–5 days and the cats have neurological signs, including tremors, especially of the muscles of the back, neck and top of head, ataxia and seizures in addition to non-specific depression and anorexia which can persist for 2–4 weeks (Fikes, 1992).

As indicated in Table 104.2, when given IV, administration of 2-PAM should be slow. Rapid IV administration can cause tachycardia, muscle rigidity, transient neuromuscular blockade and laryngospasm. At therapeutic doses, 2-PAM is generally safe and has no significant adverse effects. However, careful dosing is recommended. At high doses, 2-PAM may exhibit anticholinesterase activity including muscle weakness, ataxia, vomiting, hyperventilation, seizures, respiratory arrest and death. The LD<sub>50</sub> in dogs is 190 mg/kg (Plumb, 2005).

Patients receiving 2-PAM should be monitored for hypersensitivity reactions. 2-PAM is generally not recommended for carbamate toxicosis because AChE inhibition due to carbamates is rapidly reversible (Plumb, 2005), and 2-PAM has less affinity for carbamates than OPs (Meerdink, 2004). In addition, there is evidence that 2-PAM can reduce the protective effects of atropine in the treatment of one carbamate, carbaryl (Fikes, 1990). Since the drug is excreted by the kidneys, patients with underlying renal impairment should receive a decreased dose and be monitored closely for signs of toxicity (Plumb, 2005).

TABLE 104.2 Pralidoxime dosing in OP treatment

Species	Dosing instructions
Dogs and cats	Pralidoxime works best when combined with atropine. Pralidoxime at 20 mg/kg, 2–3 times a day. Initial dose may be given either IM or slow IV. Subsequent doses may be given IM or SQ.
Cattle	25–50 mg/kg as a 20% solution IV over 6 minutes or as a maximum of 100 mg/kg/day as a CRI IV infusion.
Horses	20 mg/kg (may require up to 35 mg/kg) slow IV and repeat every 4–6 hours.

## Cyproheptadine HCl

Cyproheptadine HCl has been successful in treating serotonin syndrome in dogs and people (Gwaltney-Brant and Rumbelha, 2002). Cyproheptadine is an antihistamine that is most commonly utilized in veterinary practice as an appetite stimulant for cats. It also is a potent serotonin antagonist (Plumb, 2005).

Serotonin is a neurotransmitter in the central nervous system. It also acts to promote platelet aggregation and as a stimulant on the smooth muscle of the respiratory, gastrointestinal and cardiovascular systems. The term serotonin syndrome is used to describe the characteristic signs which develop from excessive serotonin including autonomic, neuromuscular, behavioral and cognitive abnormalities (Gwaltney-Brant *et al.*, 2000). Excess serotonin may result from use or accidental overdose of medications that increase brain serotonin levels. These medications include selective serotonin reuptake inhibitors (SSRIs), like venlafaxine (Effexor®), paroxetine HCl (Paxil®) and fluoxetine HCl (Prozac®), as well as 5-hydroxytryptophan which is a serotonin precursor sold over-the-counter as a dietary supplement (Gwaltney-Brant and Rumbelha, 2002). See Table 104.3 for a list of medications that carry a high potential of increasing brain serotonin levels.

Dogs with serotonin syndrome typically have hyperthermia, central nervous abnormalities including tremors, seizures, ataxia, excitation or depression and hyperesthesia, and gastrointestinal effects of vomiting, diarrhea and abdominal discomfort (Gwaltney-Brant and Rumbelha, 2002). Death is possible if the signs are not controlled quickly. When used as a serotonin antagonist, the recommended dose of cyproheptadine in dogs is 1.1 mg/kg up to every 8 hours until signs do not recur (Gwaltney-Brant, 2006 personal communication). The dose can be given orally if the patient is alert, not vomiting and activated charcoal was not given within 2 hours. In those instances, the dose can be crushed, mixed with a small amount of saline and given per rectum. The drug should be discontinued after the first two doses if little or no improvement

is noted (Gwaltney-Brant *et al.*, 2000). A similar dose of cyproheptadine has also been of some benefit in controlling vocalization and disorientation in some cases of baclofen toxicosis (Wismer, 2004).

## Digoxin immune Fab

Digoxin immune Fab fragments (e.g., Digibind® from GlaxoSmithKline) are a promising treatment for life-threatening digoxin toxicosis. The Fab fragments are used as a specific antidote for digoxin since they inactivate the drug by directly binding to it. The fragments are produced by first immunizing sheep with digoxin-human albumin complexes. In response, the sheep produce antibodies that are collected, purified and cleaved with papain into Fab fragments and Fc portions (Kittleson and Kienle, 1998). Digoxin immune Fab fragments are quite expensive, so their use may be cost prohibitive and they may be difficult to obtain. A local human hospital pharmacy may be willing to sell the product to the veterinary clinic if needed.

Digoxin and other digitalis glycosides are thought to cause their effects by inhibition of the sodium-potassium ATPase pump ( $\text{Na}^+/\text{K}^+$ -ATPase) through competition with potassium for binding sites (Kittleson and Kienle, 1998). Fab fragments can actually remove a digoxin molecule that is bound to the ATPase since their affinity for digoxin is much stronger than the affinity of digoxin for the  $\text{Na}^+/\text{K}^+$ -ATPase target (Gwaltney-Brant and Rumbelha, 2002). Fab fragments may also be used as an effective antidote for digitoxin toxicosis since they bind to it, though with less affinity than for digoxin. Moreover, sufficient cross-reactivity exists with cardiac glycosides derived from *Bufo* toads and a wide variety of plants; consequently, Fab fragments may be effective at controlling toxicoses from these agents as well (Clark *et al.*, 1991; Gwaltney-Brant and Rumbelha, 2002). See Table 104.4 for a list of plants containing cardiac glycosides that Fab fragments may be effective against.

TABLE 104.3 Drugs with high serotonergic potential

Amitriptyline (e.g., Elavil®)	Lithium
Amphetamines (e.g., Adderall®)	Meperidine (e.g., Demerol®)
Clomipramine (e.g., Clomicalm®)	Moclobemide
Dexfenfluramine	Paroxetine (e.g., Paxil®)
Fenfluramine (e.g., Ponderal®)	Phenelzine
Fluoxetine (e.g., Prozac®)	Selegiline (e.g., Anipryl®)
Hydroxytryptophan	Sertraline (e.g., Zoloft®)
Imipramine (Tofranil®)	Tranlycypromine
Isocarboxazid	Tryptophan
	Venlafaxine (e.g., Effexor®)

TABLE 104.4 Plants containing cardiac glycosides

Plant species	Common name
<i>Acodkanthera oblongifolia</i>	
<i>Adonis microcarpa</i>	Pheasant's eye
<i>Asclepias physocarpa</i>	Balloon cotton bush
<i>Byrophyllum tubiflorum</i>	Mother of millions
<i>Calotropis procera</i>	King's crown
<i>Carissa laxiflora</i>	
<i>Cerbera manghas</i>	Sea mango
<i>Convallaria majalis</i>	Lily of the valley
<i>Cryptostegia grandiflora</i>	Rubber vine
<i>Helleboros.</i>	
<i>Nerium oleander</i>	Oleander
<i>Thevetia neriifolia</i>	Yellow oleander

The antidotal use of Fab fragments is indicated for treatment of potentially life-threatening cardiac glycoside toxicoses. Fab fragments should be considered when the patient has developed cardiac arrhythmias that are unresponsive to other antiarrhythmic therapy. Ideally, the appropriate dose of Fab fragments is determined by using the patient's serum digoxin level. In some cases, the laboratory at a local human hospital may be willing to perform serum levels for the veterinarian. If a serum level can be obtained, the following formula is used to calculate the number of vials (Gwaltney-Brant and Rumbeiha, 2002). This formula assumes each vial will bind 0.5 mg of digoxin:

$$\text{Dose (number of vials)} = \frac{\text{Serum digoxin concentration (ng/ml)} \times \text{Body weight (kg)}}{100}$$

In cases where the cost of the calculated number of vials is prohibitive, if digoxin levels cannot be measured, or if the Fab fragments are being used to treat another cardiac glycoside, treatment can be initiated with one to two vials and the patient observed for improvement. The reconstituted Fab fragments are administered intravenously over approximately 30 minutes. If the clinician feels the patient's clinical signs are immediately life-threatening, the product can instead be given as a bolus. Fab fragments act quickly and dramatically. The patient's cardiac status is expected to begin improving within 20–90 minutes of administration and complete resolution of the clinical effects is usually seen within 4 hours (Gwaltney-Brant and Rumbeiha, 2002). If significant improvement does not occur, additional vials may need to be given.

While monitoring the patient, it is important to note that the total serum digoxin concentration will be markedly increased with most commercial assays after Fab fragment administration. This occurs because previously tissue-bound digoxin binds to the Fab fragments, and the Fab–digoxin complexes then move into the blood to be excreted by the kidneys (Ward *et al.*, 1999). Since these patients have a compromised cardiovascular system as a result of the toxicosis, it is likely that renal perfusion is also compromised. These animals should be monitored carefully because decreased renal clearance of Fab–digoxin complexes may allow for dissociation of the toxin and recurrence of signs (Gwaltney-Brant and Rumbeiha, 2002).

The patient should also be monitored for hypokalemia. Elevated serum potassium is expected with cardiac glycoside toxicosis due to interference with the Na<sup>+</sup>/K<sup>+</sup>-ATPase pumps. After administration of the Fab fragments, potassium moves quickly back into the cells leading to a significant drop in serum potassium levels. Serum potassium levels should be checked frequently within the first few hours and hypokalemia treated if

needed (Gwaltney-Brant and Rumbeiha, 2002). The patient should also be monitored for anaphylaxis, fever and hypersensitivity reactions (Ward *et al.*, 1999).

### Antidotes for ethylene glycol: ethanol and fomepizole

*Ethanol* can be used as an antidote for ethylene glycol poisoning. Ethylene glycol is found in many products at many concentrations. Most animal intoxications occur from ingestion of antifreeze since these products may have concentrations of ethylene glycol exceeding 90%. With intoxication, the gastrointestinal, central nervous, cardiopulmonary and renal systems can be affected. Death is usually due to acute renal failure. The ethylene glycol itself does not cause the life-threatening clinical findings. It must be converted, primarily in the liver, to its more toxic metabolites including glycolic, glyoxalic and oxalic acids (Dalefield, 2004). The first step in the metabolism is conversion of the parent ethylene glycol molecule to glycoaldehyde via the enzyme alcohol dehydrogenase (ADH). Ethanol is also metabolized by alcohol dehydrogenase, therefore it competes for the enzyme, allowing time for ethylene glycol to be excreted unchanged in the urine (Mathews, 2006). Early intervention is very important to prevent the conversion to toxic metabolites. If a toxic dose was ingested and treatment is not initiated within a few hours of exposure, the patient's prognosis is grave.

The dosing recommendations for ethanol vary among authors. Intermittent dosing is possible, but a constant rate infusion (CRI) is preferred to avoid high blood concentrations of the ethanol which can exacerbate the potential clinical effects (Mathews, 2006). See Table 104.5 for examples of intermittent and CRI dosing recommendations for dogs and cats.

Ethanol is easy to obtain and is inexpensive. However, significant adverse effects will likely develop at the high doses required for treatment of ethylene glycol toxicosis, including severe respiratory and central nervous system depression and metabolic acidosis. Since the signs of ethylene glycol intoxication mirror those effects, gauging patient progress can be challenging (Plumb, 2005).

*Fomepizole*, also called 4-methylpyrazole (4MP), is marketed as Antizol-Vet<sup>®</sup> as an antidote for ethylene glycol toxicosis in dogs. Studies have found that 4MP can also be used in cats, but must be used at higher doses to be effective (Connally *et al.*, 2002). 4MP is a synthetic ADH inhibitor that works similarly to ethanol to prevent the conversion of ethylene glycol to its toxic metabolites. However, while ethanol acts by competing for the enzyme, 4MP forms a complex with ADH and its coenzyme (Connally *et al.*, 1996). Fomepizole is significantly more expensive than ethanol, but has a therapeutic advantage because it causes fewer serious side effects

TABLE 104.5 Ethanol dosing in EG treatment

	Intermittent dosing	CRI
<b>Dogs</b>	<ul style="list-style-type: none"> <li>Start with a 20% solution</li> <li>Give 5.5 ml/kg IV q4hrs for 5 treatments</li> <li>Then give 5.5 ml/kg IV q6hrs for 4 additional treatments</li> </ul>	<ul style="list-style-type: none"> <li>Start with a 5% solution</li> <li>Give at CRI rate of 5.5 ml/kg/h</li> </ul>
<b>Cats</b>	<ul style="list-style-type: none"> <li>Start with a 20% solution</li> <li>Give 5 ml/kg IV q6hrs for 5 treatments</li> <li>Then give 5 ml/kg IV q8hrs for 4 additional treatments</li> </ul>	<ul style="list-style-type: none"> <li>Start with a 5% solution</li> <li>Give at CRI rate of 5 ml/kg/h</li> </ul>

(Gaddy, 2001). Dose-related central nervous system depression is the only adverse effect expected and tends to be mild if it develops. Cats are more at risk, because they require higher doses. Since 4MP has few serious risks, treatment is recommended in cases where exposure to ethylene glycol is suspected but not confirmed (Gwaltney-Brant and Rumbeiha, 2002).

4MP is excreted in the urine and accumulates over time depending on the dosage amount and frequency of administration. Since the drug accumulates, lower doses are given following the initial dose (Gaddy, 2001). In dogs, an initial dose of 20 mg/kg of the 5% reconstituted solution is given IV. At 12 and 24 hours after the first dose, additional doses of 15 mg/kg are given. Finally, at 36 hours after the first dose, a dose of 5 mg/kg is given. In cases where the patient has not fully recovered following the above doses, additional dosing of 3 mg/kg every 12 hours is recommended (Gwaltney-Brant and Rumbeiha, 2002).

The dosing schedule in cats is an initial dose of 125 mg/kg IV, then subsequent doses at 12, 24 and 36 hours with 31.25 mg/kg (Plumb, 2005). Treatment in cats with either 4MP or ethanol must begin within 3 hours post-ingestion of ethylene glycol or prognosis is grave. In dogs, fomepizole is most effective if given within 3–6 hours of ethylene glycol ingestion; however, some benefit was seen as late as 36 hours post-ingestion. A late dose may prevent additional injury to the kidneys (Gwaltney-Brant and Rumbeiha, 2002).

## Flumazenil

Flumazenil, 1,4-imidazobenzodiazepine, is a benzodiazepine antagonist (Plumb, 2005). It is derived from the antibiotic anthramycin (Gwaltney-Brant and Rumbeiha, 2002) and blocks benzodiazepines by competition for the benzodiazepine receptor in the central nervous system

(Plumb, 2005). Flumazenil has a higher affinity for the receptor than that of the benzodiazepines; as a result, it displaces receptor-bound benzodiazepines to reverse their depressive effects (Gwaltney-Brant and Rumbeiha, 2002).

Flumazenil has been used as a successful therapy for benzodiazepine toxicosis in dogs and cats (Wisner, 2002; Plumb, 2005). It is also used to improve neurological signs in dogs with severe hepatic encephalopathy (Plumb, 2005). Flumazenil has been used in humans, with some success, to reverse ethanol and tegretol-induced central nervous system depression. Consequently, it is reasonable to assume flumazenil also could be effective in small animal patients in such cases (Gwaltney-Brant and Rumbeiha, 2002).

The recommended flumazenil dose in dogs and cats is 0.01–0.02 mg/kg administered by rapid IV injection (Plumb, 2005). The medication rapidly crosses the blood–brain barrier and a swift reversal of benzodiazepine-induced sedation is expected within 1–2 minutes (Gwaltney-Brant and Rumbeiha, 2002). The dose may need to be repeated multiple times, since flumazenil's half-life is shorter than most of the benzodiazepines. The half-life of flumazenil is only 1 hour in humans (Plumb, 2005), but the half-life of diazepam in the dog is 6 hours. The patient should be monitored carefully for recurrence of signs and additional doses of flumazenil given as needed. Repeat doses should not be given in asymptomatic patients. Flumazenil may act as a benzodiazepine agonist if administered at high doses despite its antagonist action at therapeutic doses (Gwaltney-Brant and Rumbeiha, 2002).

Flumazenil is a costly medication and carries the risk of significant adverse effects. It should be reserved for cases where the patient has life-threatening benzodiazepine-induced clinical signs. The drug should be given through a patent IV catheter, because extravascular leakage can cause extensive local tissue irritation and necrosis (Gwaltney-Brant and Rumbeiha, 2002). In humans, vomiting, cutaneous vasodilation, vertigo, ataxia and blurred vision have been reported following flumazenil administration (Plumb, 2005). Seizures and death are also rarely reported in humans after treatment with flumazenil (ASHSP, 2003).

Flumazenil may lower the seizure threshold and is contraindicated in cases when seizures are anticipated. It also may increase intracranial pressure and should not be used in patients with head trauma (Gwaltney-Brant and Rumbeiha, 2002). Additionally, flumazenil is contraindicated in patients with life-threatening tricyclic antidepressant toxicosis and should be used only after careful consideration in cases of multiple drug overdose (Plumb, 2005). Most human deaths and seizures associated with flumazenil followed use in cases of tricyclic antidepressant toxicosis (ASHSP, 2003) and mixed overdoses (Plumb, 2005).



## Lipid emulsion

Intravenous infusion of a lipid emulsion (ILE) has been used successfully in humans to treat intoxication with local anesthetic medications and the antidepressant bupropion (Crandell and Weinberg, 2009; O'Brien *et al.*, 2010). Research studies in dogs, rats and rabbits have shown ILE to be effective in managing bupivacaine, verapamil, propranolol and clomipramine toxicosis. ILE has also been used in the treatment of companion animal intoxications as shown in two recent case reports. In one, ILE was used to treat lidocaine intoxication in a cat and in the other it was used to treat moxidectin toxicosis in a puppy.

Lipid emulsions are made from purified soybean oil in water, are commonly used in medicine to provide intravenous (parenteral) nutrition and are the delivery mechanism for certain hydrophobic drugs like propofol. The mechanism by which ILE is effective at treating toxicoses is not yet fully understood. However, the fact that ILE seems to be most effective in treating overdosage of lipid-soluble medications suggests that the infusion expands the amount of plasma lipid which acts as a sink in which the offending drug can gather reducing free drug concentrations. In theory, the drug is trapped in the plasma lipid so it is not available to act on other tissues (Crandell and Weinberg, 2009; O'Brien *et al.*, 2010).

The APCC uses the following dosing protocol. Using a 20% product, give an initial bolus of 1.5ml/kg slowly then start a continuous rate infusion (CRI) of 0.25ml/kg/min for 30–60 minutes. Four hours after the CRI is finished, check the serum for hyperlipemia and to see if the serum is orange or yellow. If the serum looks normal, repeat the initial bolus and CRI again. If hyperlipemia or a color change is present, check the serum for resolution every 2 hours. Repeat the initial bolus and CRI once the hyperlipemia or color change resolves. If a third dose is needed, follow the above directions beginning 4 hours after the second CRI finishes. Do not give more than three doses if there has been no significant response.

ILE may be effective in treating intoxications with many lipid-soluble medications including lidocaine, moxidectin, bupropion, bupivacaine, levobupivacaine, ropivacaine, verapamil, propranolol and clomipramine. Possible side effects of administering ILE include induction of pancreatitis, creation of a fat embolism, immunosuppression, phlebitis, thrombosis, hypertriglyceridemia and hepatic lipidosis (Crandell and Weinberg, 2009; O'Brien *et al.*, 2010).

## Methocarbamol

The skeletal muscle relaxant methocarbamol has proved to be very useful in the management of severe muscle

fasciculations, tremors and seizures associated with a variety of toxicologic agents. The exact mechanism by which methocarbamol works is unknown, but it is thought to act centrally to block nerve impulses in the brain stem, spinal cord and subcortical levels of the brain (Gwaltney-Brant and Rumbeiha, 2002; Plumb, 2005).

Methocarbamol has been used successfully in veterinary medicine to treat the following types of toxicoses: permethrin in cats (Richardson, 2000a), metaldehyde (Richardson *et al.*, 2003), strychnine (Gwaltney-Brant and Rumbeiha, 2002; Plumb, 2005) and tremorgenic mycotoxins (Schell, 2000). It is able to reduce the severity of tremors without causing the severe CNS depression often encountered when using a barbiturate medication (Gwaltney-Brant and Rumbeiha, 2002).

The dosage for dogs and cats is 55–220mg/kg administered slowly IV at a rate of no more than 2ml/min (Schell, 2000). Methocarbamol administration should be repeated if the signs recur; however, the total dose should not exceed 330mg/kg in a 24-hour period to avoid central nervous system and respiratory depression (Gwaltney-Brant and Rumbeiha, 2002; Richardson *et al.*, 2003). The dosage for horses is 15–25mg/kg by slow infusion (Plumb, 2005). The drug should not be given SQ and extravasation of methocarbamol should be avoided as the solution can be irritating (Plumb, 2005).

In dogs and cats, adverse effects may include sedation, salivation, emesis, lethargy, weakness and ataxia. Slow administration of the drug can help to avoid salivation and emesis. Sedation and ataxia can be seen in horses following methocarbamol administration. This medication is contraindicated in animals intended for food purposes and in patients hypersensitive to it. The manufacturer lists known or suspected renal insufficiency as a contraindication to injectable methocarbamol therapy since the injectable product contains polyethylene glycol 300, which has been found to increase acidosis and urea retention in renal-impaired humans (Plumb, 2005). For this reason, veterinary patients treated with injectable methocarbamol should receive IV fluid support and have kidney values monitored if renal impairment is suspected or is a possible outcome of their clinical signs. For example, patients with prolonged tremors or seizures can release muscular myoglobin. The myoglobin is excreted by the kidneys and can lead to renal damage (Volmer, 2004).

## Naloxone HCl

Naloxone HCl (Narcan®) is an injectable opiate antagonist used to reverse the effects of opiate medications. It is able to reverse opioid agonist/antagonists such as butorphanol as well. This drug is also being investigated for treatment of other conditions like septic, hypovolemic and cardiogenic shock (Plumb, 2005).

Naloxone is a pure opiate antagonist, and it has no analgesic activity or other agonist effects. Although the exact mechanism for its activity is not fully understood, it is thought the drug acts as a competitive antagonist by binding to multiple opioid receptor sites. The drug apparently has its highest affinity for the *mu* receptor which makes it an ideal antagonist since most of the clinically useful opioids work by binding to the *mu* receptor (Plumb, 2005; Volmer, 2006).

Naloxone reverses most of the effects of opioids including analgesia and respiratory and central nervous system depression (Plumb, 2005). It does not reverse the emetic actions of apomorphine in dogs (Volmer, 2006). At high doses, naloxone increases dopamine levels and acts as a GABA antagonist (Plumb, 2005).

Because orally administered naloxone is only minimally absorbed and the drug is destroyed rapidly in the digestive tract, an injectable formulation is preferred. The onset of action is normally within 1 to 2 minutes when given IV and within 5 minutes when given IM. The duration of action is usually 45–90 minutes but may be as long as 3 hours. Since the duration of action of naloxone may be shorter than the medication being reversed, additional doses may be required (Plumb, 2005).

The recommended dosage for dogs is 0.04 mg/kg IV, IM or SC; for cats, 0.02–0.04 mg/kg IV and for horses, 0.01–0.02 mg/kg IV (Plumb, 2005). In patients that have been intentionally given an opioid to treat a painful condition, abrupt reversal can lead to tachycardia, vasoconstriction and hypertension. In these cases, the unwanted central nervous system and respiratory depression can be reversed without reversing the analgesia. To achieve this, dilute 0.04–0.1 mg of naloxone in 5–10 ml of normal saline and give slowly to effect by dosing 0.5–1 ml each minute (Mathews, 2006).

Naloxone is considered quite safe at therapeutic doses. Very high doses have been associated with seizures in a few patients perhaps due to GABA inhibition. It is contraindicated in patients that are hypersensitive to it and should be used with caution in animals with pre-existing cardiac abnormalities due to the potential cardiovascular adverse effects (Plumb, 2005).

## N-acetylcysteine

N-acetylcysteine (NAC) is used to prevent methemoglobinemia and hepatic necrosis in acetaminophen (APAP) toxicosis. APAP is metabolized in the liver primarily via glucuronidation and sulfation to non-toxic metabolites. However, other pathways of metabolism exist which yield the toxic metabolites N-acetyl-para-benzoquinoneimine (NAPQI) and para-aminophenol (PAP) (Aronson and Drobatz, 1996; McConkey *et al.*, 2009). In a significant APAP exposure, the glucuronidation and sulfation

pathways become saturated, leading to increased production of NAPQI and PAP. NAPQI is produced through the P450 mixed function oxidase system and is normally inactivated through conjugation with glutathione (Richardson, 2000b). PAP is produced by deacetylation and is removed both through conjugation with glutathione and by *N*-acetylation. Dogs and cats are deficient in the enzymes needed for *N*-acetylation (McConkey *et al.*, 2009).

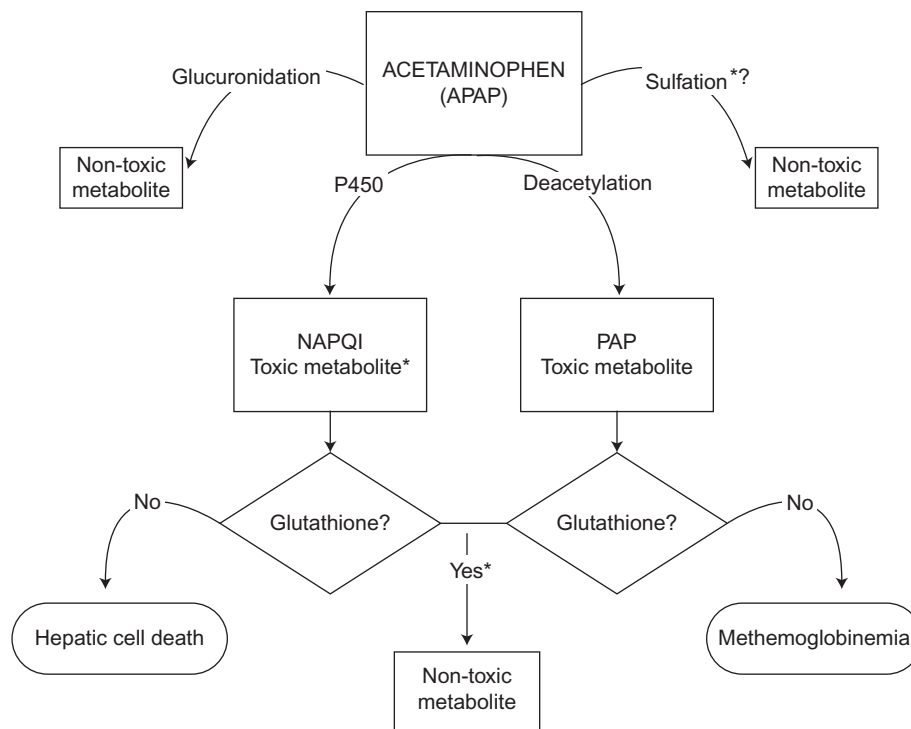
Initially the excess NAPQI and PAP will be detoxified through conjugation with glutathione. However, once glutathione stores are depleted, the NAPQI metabolite can lead to liver necrosis and the PAP metabolite can lead to methemoglobinemia. NAPQI binds to hepatic cell membranes causing oxidative injury to the bi-lipid layer and potential cellular death and necrosis (Richardson, 2000b). PAP causes redox cycling in the red blood cells (RBCs) leading to oxidation of the hemoglobin to methemoglobin which is unable to carry oxygen (McConkey *et al.*, 2009). While all species can develop both hepatic and RBC effects, dogs more commonly develop hepatic necrosis, whereas cats more commonly develop methemoglobinemia and Heinz body anemia (Taylor and Dhupa, 2000).

There are at least two mechanisms by which NAC prevents methemoglobinemia and hepatic necrosis from APAP. NAC binds directly with NAPQI rendering it inactive and therefore non-toxic. NAC is a precursor for glutathione production. It restores and maintains glutathione levels by increasing synthesis (Richardson, 2000b). NAC administration may also lead to increased serum sulfate which could re-establish the sulfation pathway to non-toxic metabolites (Aronson and Drobatz, 1996) (Figure 104.1).

The therapeutic dose of NAC for dogs and cats is a loading dose of 140 mg/kg PO or IV (as a 5% solution), then 70 mg/kg PO or IV every 6 hours for seven treatments. The number of additional treatments varies between authors; some recommend up to 17 treatments (Plumb, 2005). Presently, both an injectable form and oral solution are on the market. When given orally, NAC can cause gastrointestinal irritation so proper dilution is important.

## Pamidronate disodium

Pamidronate disodium (Aredia®) is a biphosphonate used in the treatment of hypercalcemia associated with malignancy and vitamin D<sub>3</sub> toxicity in dogs (Plumb, 2005). Vitamin D<sub>3</sub>, also called cholecalciferol, is found in some rodenticides. Calcipotriene, which is an analog of cholecalciferol, is found in some human prescription topical antipsoriasis medications (e.g., Dovonex®) (Pesillo *et al.*, 2002). Since vitamin D<sub>3</sub> plays an important role



**FIGURE 104.1** The metabolism of acetaminophen (APAP). Areas of *N*-acetylcysteine (NAC) action are indicated with an \*.

in calcium and phosphorus homeostasis, oral exposure to these rodenticides or topical medications can lead to significant elevations in serum calcium and phosphorus levels. Vitamin D<sub>3</sub> enhances calcium and phosphorus absorption from the GI tract. In addition, it works with parathyroid hormone to promote calcium reabsorption in the kidney and mobilization of calcium from the bone via osteoclastic-mediated bone resorption (Rumbeiha *et al.*, 2000; Morrow, 2001).

Calcium maintains cell membrane stability and is a second messenger in several cellular responses (Pesillo *et al.*, 2002). Therefore, increasing serum calcium can cause many cellular effects including altered cell membrane permeability, a decrease in cellular energy production and cellular necrosis (Morrow, 2001). Patients poisoned by vitamin D<sub>3</sub> and its analogs often present with polyuria, polydipsia, vomiting, anorexia, lethargy, hypercalcemia and hyperphosphatemia (Pesillo *et al.*, 2002). If elevations in serum calcium and phosphorus concentrations go unchecked, the plasma calcium phosphorus product (Ca X P) can rise above 60, leading to soft tissue mineralization (Morrow, 2001). Mineralization of the kidneys and heart can lead to a life-threatening decrease in function of these organs.

Pamidronate acts to decrease serum calcium levels by binding to hydroxyapatite crystals in the bone to prevent their dissolution and bone resorption. It also interrupts osteoclast activity and induces osteoclast apoptosis (Plumb, 2005). Some authors suggest pamidronate may

decrease intestinal absorption of calcium as well (Pesillo *et al.*, 2002).

The recommended dose of pamidronate is 1.3–2 mg/kg given as a slow IV infusion over several hours (Plumb, 2005). Pamidronate must be given in saline and should not mix with any IV fluid containing calcium (e.g., Ringer's). In most cases, a single dose will be effective in lowering calcium levels back to normal (Gwaltney-Brant and Rumbeiha, 2002). However, some patients may require an additional dose 5–7 days after the initial treatment (Morrow, 2001). Calcium levels should be monitored once daily for at least 10 days after they have returned to normal. Pamidronate is most effective if given within 24–36 hours of the exposure, prior to development of soft tissue mineralization (Gwaltney-Brant and Rumbeiha, 2002).

Anemia, thrombocytopenia and granulocytosis have been reported in humans following pamidronate administration. In dogs, hypersensitivity reactions, electrolyte abnormalities including hypomagnesemia and hypocalcemia, arrhythmias and renal toxicity are possible (Plumb, 2005). Slow infusion of the drug over at least 2 hours may help in avoiding renal effects. Use of pamidronate in dogs with impaired renal function is controversial since it may confound renal injury (Gwaltney-Brant and Rumbeiha, 2002).

Pamidronate is expensive, but its use may be more cost effective than using salmon calcitonin in vitamin D<sub>3</sub> toxicosis. Pamidronate lowers plasma calcium

concentrations within 24–48 hours of a single IV dose. Calcitonin requires dosing several times daily due to its short half-life. Additionally, with calcitonin therapy, saline diuresis and treatment with diuretics and corticosteroids must also be performed, requiring many days, sometimes multiple weeks, of hospitalization. In most cases treated with pamidronate, treatment can be performed on an out-patient basis once the calcium has returned to the normal range (Morrow, 2001; Pesillo *et al.*, 2002). Pamidronate is also preferred to calcitonin since patients may become refractory to calcitonin treatment after 10 days of therapy (Rumbeiha *et al.*, 2000; Morrow, 2001).

### Phytonadione (vitamin K<sub>1</sub>)

Phytonadione, a naphthoquinone derivative identical to naturally occurring vitamin K<sub>1</sub>, is used in the treatment of anticoagulant toxicosis resulting from anticoagulant rodenticides, the medication warfarin or moldy sweet clover (Plumb, 2005). All household pets, including pocket pets, may be exposed to anticoagulant rodenticides and the medication warfarin. Livestock and horses are more likely to be poisoned by the dicoumarol in moldy sweet clover, but they can also be exposed to rodenticides (Knight, 2004).

Anticoagulants block clotting factor activation. Vitamin K<sub>1</sub> is involved in the activation of precursor clotting factors into functional factors. During the activation, vitamin K<sub>1</sub> is converted to inactive vitamin K<sub>1</sub> epoxide. Normally, vitamin K<sub>1</sub> epoxide is reactivated by vitamin K<sub>1</sub> epoxide reductase. Anticoagulants work by inhibiting this enzyme and halting the recycling of vitamin K<sub>1</sub> (Merola, 2002). As a result, the number of active clotting factors decreases.

The vitamin K<sub>1</sub>-dependent clotting factors are II, VII, IX and X. These factors are involved in all three pathways of the coagulation system: the extrinsic, intrinsic and common (Means, 2004a). Active bleeding is not expected for 3 to 7 days after the ingestion because the body has a reserve of active clotting factors. When these degrade naturally or are consumed, clinical signs are possible. If an external source of vitamin K<sub>1</sub> is provided, the factors will be activated normally (Merola, 2002).

Bleeding can occur anywhere within the body and the presenting clinical signs will relate to where the bleeding has occurred. The signs may be vague and non-specific. In dogs, dyspnea, coughing, lethargy and hemoptysis are commonly reported due to bleeding into the chest cavity (Merola, 2002). With moldy sweet clover poisoning in livestock, prolonged bleeding in some members following routine surgical procedures, like castration or dehorning, is often the first indication of a problem within the herd (Knight, 2004).

The dose for vitamin K<sub>1</sub> in household pets is 1.5–2.5 mg/kg orally twice daily. Pocket pets and other small patients can be given the injectable formulation orally if a suitable tablet size is not available. Small patients should be dosed at the high end of the range and larger patients (e.g., large breed dogs) should be started at the low end (Means, 2004a). Vitamin K<sub>1</sub> should be given with a fatty meal to enhance absorption. In dogs, giving the medication with canned food caused a 4–5-fold increase in bioavailability (Plumb, 2005).

Oral administration is preferred since the vitamin K<sub>1</sub> will be carried, via the portal circulation, directly to the liver where the activation of clotting factors occurs (Merola, 2002). Additionally, vitamin K<sub>1</sub> injections have been associated with anaphylactic reactions and hematoma formation (Plumb, 2005). However, IM administration is recommended by many authors for livestock, perhaps for ease of administration. The recommended dose for cattle, horses, swine, sheep and goats is 0.5–2.5 mg/kg IM (Knight, 2004; Plumb, 2005).

The length of vitamin K<sub>1</sub> administration depends on the toxicant. Warfarin exposures generally require a 14-day course. Second generation anticoagulant rodenticides should be treated for 30 days, except for bromadiolone which should be treated for 21 days (Means, 2004a). Moldy sweet clover poisoning should be treated for 1–2 weeks (Knight, 2004). In all cases, a prothrombin time (PT) should be checked 48–72 hours after the course of vitamin K<sub>1</sub> is complete. If this test is abnormal, resume vitamin K<sub>1</sub> therapy for an additional 7 days (Merola, 2002).

Blood transfusions may be required in severely symptomatic patients, since it may take 6–12 hours for the liver to produce active clotting factors following initiation of vitamin K<sub>1</sub> therapy (Plumb, 2005). Exercise restriction is indicated in all patients during this lag time to prevent active bleeding (Merola, 2002). Substituting vitamin K<sub>3</sub> (menadione) for vitamin K<sub>1</sub> is not recommended, as it is not as effective and is nephrotoxic in horses (Merola, 2002; Knight, 2004).

## CONCLUSION

Proper client education is crucial to poison prevention. However, when poisonings do occur, the clinician should concentrate on treating the patient and not the poison. The same principles of history collection, physical examination and patient monitoring that are used in other cases should be applied when managing a poisoning patient. Appropriate decontamination and antidotal therapies should be decided on after initial patient stabilization.



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